

What to do about scientific misconduct

A meeting intended to sustain the fight against misconduct in science should look for ways of making academic institutions more responsible and for weakening the links between personal success and publication.

NOT before time, the National Academy of Sciences in the United States and its sister organizations in medicine and engineering have stirred themselves on the issue of scientific misconduct. Next week, they plan a public meeting in Washington at which members of the scientific community will have their say. The occasion for the meeting is said to be the second anniversary of a report called *Responsible Science*, which drew an unremarkable distinction between "misconduct in science" (fraud) and "questionable research practices". To judge from a statement by the president of the National Academy and his two colleagues (*Proc. natn. Acad. Sci. U.S.A.* **91**, 3479–3480; 1994), next week's meeting is meant to give the research and academic community a further push towards vigilance against misconduct as well as to persuade US government agencies to adopt common definitions and policies in this field.

But the academy has a responsibility to take a more active and continuing role in supervising the conduct and the manners of the scientific community in the United States, both in general and in particular. The steady stream of misconduct cases in the past 15 years has corroded the civility of the US scientific enterprise and undermined its public reputation. The academy themselves have a vivid interest in halting this process. In some respects, the distressing events of the recent past have shown that no body other than the academy can do some of the things now required.

Complaint

Misconduct in science, as in other professions, comes in all shapes and sizes. It ranges from the flagrant and persistent fabrication of data (J. Darsee, Emory and Harvard, 1981) to proven plagiarism (J. Felig & R. Suliman, Yale, 1984) to the obstinate defence of an indefensible paper (T. Imanishi-Kari & D. Baltimore, Tufts and Whitehead Institute, 1986). Recognizable cases such as these are embedded in the steady background of complaint by working scientists that their ideas have been misappropriated or insufficiently acknowledged or that rivals have unfairly over-interpreted preliminary data so as to jump to a conclusion only afterwards confirmed (C. Rubbia, CERN, 1987). Recent history is so peppered with allegations of this kind ("questionable research practices") that outsiders can be forgiven for believing that science is not the honourable profession it pretends to be, but a mafia in which rival gangs scheme to win the credit for intellectual innovation.

The first need is for a sense of proportion, which the

academy could provide. Those who doubt that need should reflect on what happens in other professions. Stealing other people's money is regrettably commonplace, but ordinary burglary does less damage to the collective pocket-book than thefts by people whose profession is the provision of financial services. Although most of the \$100 billion or so needed to rescue the US savings and loan industry may be attributable to naive misjudgement by people who should have known better, much of it is attributable to dishonesty.

Theft

The recent record of financial fraud is spectacular. The collapse of the bank called BCCI two years ago will probably be shown to have involved the theft of some \$2 billion by people who may yet be identified and convicted. People selling suspect bonds from offshore tax havens pop up repeatedly, and do good business until they are rumbled. Even apparently respectable corporations engage in the theft of other people's money, witness the fines regularly levied on insurance companies in the United States and Europe for selling inappropriate insurance policies to inexperienced customers. Yet the punishment for white-collar theft rarely matches the gravity of the offences. Mr Bernie Cornfeld, a 1960s pioneer of the sale of worthless bonds, has successfully evaded US prosecutors ever since. Mr Robert Maxwell, even more openly a thief, drowned at sea before the law caught up with him. In Britain, there is still indignation that a financier convicted of selling worthless securities worth £27 million was sentenced only to a spell of community service.

Casualty

That sorry record should not be mistaken for a claim that, by comparison with what happens with money, the consequences of misconduct in science are unimportant. Rather, it is a proof that they are different — different in kind. And while individuals may occasionally be damaged by acts of misconduct, the chief casualty is science itself, and in several ways. First, the public reputation of the most daring and important branch of contemporary scholarship, whose declared objective is to define the truth (with appropriate Popperian qualifications), is damaged. Second, the education of young people is hindered by the shabby example they are offered. And, to the extent that misconduct results in spurious records in the literature, the time and energy of other productive people is wasted, even stolen. Those are all matters that affect the conduct of science. The world's



academies cannot stand apart from them.

There are also practical reasons why the US academy should become engaged. The arrangements evolved in the United States for investigating misconduct are at best hamfisted and ineffective. That is especially the case in the biomedical field, where most of the trouble has been. A decade ago, the US National Institutes of Health (NIH), in haste and with too little consideration, set up the Office of Scientific Integrity (OSI) within its own administration. The purpose was to investigate allegations of misconduct and to adjudicate on them, recommending administrative penalties (such as exclusion from grant awards) where appropriate. Explicitly, OSI was not intended as a legal tribunal, but as a means of arriving at 'the truth'; members of its staff were fond of likening their methods to those of a scientific investigation, not a legal process. OSI might have lasted longer if it had acted more like a judge than a prosecutor, but its days were numbered from the beginning because its procedures were innocent of due process. When the penalties of an adverse finding might include a ruined reputation and the loss of livelihood, any half-competent lawyer could have told it that contentious cases would end up in the courts.

OSI's successor, the Office of Research Integrity (ORI) within the Department of Health and Human Services, has not been conspicuously more successful. The prosecution briefs inherited from OSI against Mikulas Popovic and Robert Gallo came to grief because of a hearing before the department's Appeals Board at the end of last year. (The Popovic case was dismissed for want of evidence, when that against Gallo was petulantly withdrawn in the expectation of a similar outcome.) Meanwhile, Imanishi-Kari still awaits a verdict (see *Nature* **358**, 177; 1992). ORI could, no doubt, rewrite its rules so as to guarantee those accused the full panoply of due process, but that would be tantamount to the creation of a new system of federal courts for the prosecution of misconduct in biomedical research alone. The Department of Justice would hardly stand for that.

Lesson

The first lesson to be learned from this brief history is that neither judicial nor quasi-judicial processes are an effective means of settling allegations of misconduct. The obvious difficulty, in the United States, is that the Constitution impels them in that direction. Any administrative decision by a federal agency will be open to appeal in the courts if it can be held that due process may have been denied. Moreover, it is a criminal offence to lie to the US government, as for example in an application for public funds to support research, but a misstatement is a lie only if the person who made it can be shown to have intended to deceive. Taken together, these difficulties explain why OSI and ORI have brought only the narrowest of indictments against those who now believe themselves to be its victims — and have nevertheless lost their biggest cases.

But, if not the courts, then who should monitor conduct? The simple answer is what it has been all along: the institutions at which people work. Unfortunately, experience has shown that universities and research institutes have been less

than zealous in that regard. They wish to think the best of apparently talented members of their staffs, and fear the damage that will be done to their reputations if they are discovered to be less than excellent in any way. Their investigations of allegations of misconduct have too often, as a consequence, been so much whitewash. There is an urgent need for some incentive to make institutions more inquisitive and rigorous. One way of doing that would be to levy administrative fines on institutions whose investigations are judged inadequate.

Remedies

Such an arrangement would have several advantages. First, it would weaken but not entirely sever links between individuals and the courts. Second, it would keep institutions on their toes; the prospect that slackness might mean that \$100,000 or so is snatched away would be a powerful spur to vigilance. Third, it would have the effect of making institutions more vigilant in stamping out "questionable research practices". To make institutions liable to administrative fines would of course require that they, rather than individuals, should be the titular holders of research grants, but that change would be mostly a formality (and would bring US practice into line with that in most other parts of the world). And who would judge whether institutions' investigations of misconduct cases were satisfactory? Only the academy has the understanding and the prestige for such a task.

That, however, does not complete the list of what needs doing. Next week's meeting should pay at least some attention to understanding the causes of the rash of misconduct in recent decades. (Extra vigilance and changing conventions, as on co-authorship, no doubt accounts for much of it.) The most important cause is the pressure to which working scientists are subjected to be seen to publish regularly and often. When promotions, appointments and the award of research grants hang as crucially as at present on people's publication records, it is inevitable that they will try to magnify their reputations by publishing more than they need (and have to say). That is also the explanation of people's eagerness to be co-authors of articles of whose content they are largely ignorant (and whose conclusions they are unable to defend), and of the inadequate and even misleading attributions of credit for earlier work now commonplace in the literature.

It would be unfortunate if next week's meeting does not pay some attention to this issue, which is not to suggest that it can be easily resolved. Deliberately weakening the link between publications and the advancement of careers in research would amount to a transformation of the present culture. But the academy should make a study of the question, looking for ways in which the link between publication and personal success could be at least attenuated. The benefits of that would be not merely to diminish the rash of "questionable research practices", but also to make the scientific profession more seemly and the scientific literature more reflective and thus more intelligible. Those are also objectives that should be close to the academy's heart. □

Europe draws up plans for a major programme of Moon exploration

Paris and Washington. Twenty-five years after the United States captured the imagination of the world with the Apollo Moon landings, the European Space Agency (ESA) is preparing to ask its space ministers to approve a long-term programme of expeditions designed to explore, exploit and colonize the Moon.

The agency hopes that such a 'return to the Moon' programme, which ministers will be asked to consider for funding next year, will rekindle international support for space exploration. The final phase could, as now planned, include an artificial ecosystem and a permanent manned presence.

ESA is presenting its proposal as a long-term, open-ended venture. To make the programme appear less daunting and allow greater budgetary flexibility, the agency, based in Paris, has also designed it in four overlapping phases.

If funding is approved, the first 'exploratory' phase could begin within a decade. This would involve sending orbiting lunar observatories, such as the proposed MORO

mission (see *Nature* 369, 90; 1994), to survey the surface and interior of the Moon over several years, using a wide range of remote sensing technology.

According to Julie Cave, an astronomer

at University College London and a member of the MORO science team, this would be much more comprehensive than any previous survey, including that carried out recently by the US Clementine observer (see below). Planetary scientists, she adds, have become "immensely interested" in a new Moon mission following the preliminary data obtained from Clementine.

The observatories would also survey lu-

nar resources and identify suitable sites for landing craft, as well as building a manned outpost on the far side of the Moon. New technology such as rovers would also be tested during this phase.

The second phase — a 'permanent robotic presence' — would establish the Moon as a platform for automatic large scientific instruments and telescopes. Such instruments would take advantage of the Moon's stable surface, its lack of an atmosphere and the absence of electromagnetic interference emitted from Earth on the 'radio-quiet' far-side.

Astronomers say the Moon offers unique opportunities for ultraviolet and submillimetre imaging, and for very-low-frequency astronomy. It would also provide improvements measured in orders of magnitude in angular resolution and sensitivity at all wavelengths, they say.

In the third phase, the 'utilization of lunar resources', robots would search for geological resources and seek ways to exploit them for use in establishing a manned outpost in the fourth phase.

ESA also says it should be feasible to manufacture propellants *in situ* for refuelling craft returning to Earth. This phase would also study the possibility of excavating an underground lunar base. Astronauts would be required to visit occasionally during this phase.

The final phase would involve establishing the first artificial ecosystem and permanent manned presence on another celestial body. This would provide the groundwork for exploration to Mars and beyond.

At a time when Europe is repeatedly scaling down its space plans because of its budgetary difficulties much of this may sound like science fiction. But officials in both Germany and France, for example, say that the proposals could provide a much-needed opportunity to look beyond immediate pre-occupations and define a long-term space strategy.

At the same time, however, German officials add that the proposal is unlikely to receive support until Europe resolves outstanding budgetary problems, in particular the terms of its participation in the National Aeronautics and Space Administration (NASA)'s international space station.

The United Kingdom is less enthusiastic. British space officials point out that they are mainly interested in short-term commercial space applications and 'cost-effective' science missions, and that ESA's budget ►



Heading back? NASA's micro-rover in New Mexico — (see below).

US military backs new lunar mission

Washington. Draft proposals from the European Space Agency for a long-term Moon programme (see above), combined with growing enthusiasm among the American military for a follow-up to the recent Clementine lunar mapping mission, may revive interest in lunar exploration at NASA.

NASA has abandoned its own lunar ambitions. Two years ago the US Congress killed the agency's plans for a pair of US\$ 100 million lunar orbiters, and forced it to close the exploration office that was studying a return of man to the Moon.

The little momentum which exists for a US return to the Moon is coming from the Department of Defense (DoD). Although Clementine was designed to test hardware, and not as a science mission, it has filled the void in lunar exploration left by NASA.

Members of the military team which organized the Clementine mission are now lobbying for a Clementine 2 to test advanced propulsion and communications technologies not flown on the first mission.

They also argue that Clementine 2 could carry a rocket-propelled lander, based on hardware developed in the Pentagon's Strategic Defense Initiative (SDI). The lander, which has been built by the Air Force's

Phillips laboratory in New Mexico, recently deployed a two-pound micro-rover on a simulated lunar landscape.

The US\$100 million Clementine project is considered to have been a success, despite the software failure that has made it unlikely that the craft will rendezvous with an asteroid this summer as was planned.

The project has shown that deep space missions can be put together quickly and cheaply. Advocates argue that demonstration projects such as Clementine 2 are needed to recover some of the massive investment in advanced spacecraft technology made during the SDI programme.

Such advocates, who hope to obtain funding for a new mission from the Air Force or other armed forces, believe its approval will depend on persuading NASA to share the costs of the project and to provide the reasons for landing on the Moon. They hope, for example, that NASA will agree to use the craft to test advanced lightweight scientific instruments for planetary exploration.

But NASA has had to watch from the sidelines as the DoD earned the accolades for Clementine, and it is now eager to prove that it too can mount cheap and clever missions.

Tony Reichhardt

is already stretched.

But the officials agree that they might become interested if the costs of the programme were shared with international partners. "We are certainly not dismissing it [the proposal]," says one official in London.

Indeed, ESA agrees that its plans are only to be achieved by a global programme. Next week, the agency will host a workshop in Switzerland on plans to study and explore the Moon. The meeting will also be attended by representatives of NASA, as well as of the Russian, Japanese and European space agencies.

Some NASA scientists, conscious that Congress would reject any plan for a NASA Moon mission, are beginning to talk of the ESA proposal as a way back into action. They believe it could revive flagging US interest in the Moon.

Wendell Mendell of NASA's Johnson Space Center says that the ESA proposal is "totally independent of NASA, which to me is good news". He adds that the US Congress is likely to accept a lunar exploration plan only "if it is brought to the table by an external party".

Jean-Jacques Dourdain, associate director for strategy, planning and international policy at ESA, describes the Moon as a "suburb" of Earth, and says that a lunar mission is inevitable at some point. He argues that the time is now right for space agencies to recapture the momentum that has been lost in space exploration.

Dourdain adds that ESA will not oversell the potential industrial spin-offs of a Moon mission — a mistake he says was made with attempts to use microgravity research, which has been used to "sell" the space station. He says that ESA is keen to assemble a "credible" plan that it can present to its member states and potential foreign partners.

Declan Butler & Tony Reichhardt

Switzerland eyes the costs of joining Framework

Basel. Swiss scientists are growing increasingly nervous that their government's enthusiasm for participating in the European Union (EU)'s fourth five-year Framework programme may take money away from basic research in order to cover the higher-than-anticipated costs that its participation would involve.

Last week, the foreign ministers of the EU announced in Brussels that bilateral negotiations with Switzerland will start "as soon as possible". Officials in Berne expect that the next research ministers' meeting at the end of June will open the way for bilateral talks about Framework.

Initial discussions took place two years ago, in the context of Switzerland possibly becoming a member of the European Economic Area (EEA). Since the beginning of this year, countries in the EEA — such as Sweden, Austria and Finland — have automatically participated in Framework.

Although Swiss voters rejected the EEA proposal at the time in a referendum, they approved the idea of participating in Framework. If parliament agrees, delegations from Berne and Brussels will meet later this year to start talks on a bilateral agreement for Swiss participation in European research programmes.

But calculations of its potential financial commitment were based on the ECU6.6 billion costs of the third five-year Framework programme (1989–93). In December 1992 the Swiss parliament approved SF477 million for participation in European research programmes for a four-year period.

In contrast, the budget for the fourth Framework will be ECU12 billion (the increases result primarily from additional European Commission projects). This will mean an expected cost to Switzerland of SFr800 million. And the need to find an additional annual SFr60–70 million on top of funds already approved by the parliament comes at a time when the federal government's finances are already tight.

Jean-Pascal Delamuraz, the Swiss Economic Minister, says that a definite solution has not yet been reached. "We will see whether it is really necessary to pay so much to take part in EU programmes and whether we will have to sacrifice something."

Rather than reduce its level of participation — which would need negotiating with Brussels — the government would prefer to find the extra money from research funds that would otherwise have been spent inside Switzerland.

Tim Guldemann, of the Gruppe für Wissenschaft und Forschung, which advises the Swiss government on research and science policy, says that about half the outstanding amount would have to be found from other budgets. But he points out that, providing Swiss researchers are successful in applying for grants from Brussels, more than SFr200 million will be returned to the country each year.

Nevertheless, many Swiss researchers fear that participation in European programmes could lead to reduced funding for basic research in Switzerland itself. Most research administrators agree in principle that the 'compensation' solution is appropriate, but no-one is eager to see his or her budget cut.

Hans Peter Hertig, general secretary of the Swiss National Science Foundation, the state agency that funds basic research, says he supports Swiss participation in European-level programmes funded through Brussels, but not at the price of a reduction in basic research at home.

"The fourth framework is mainly about applied research carried out in collaboration with industry", says Hertig. He argues that money earmarked for applied research in Switzerland might more suitably be transferred to finance the European participation, and that funding for basic research should be protected.

Paul-Erich Zinsli, vice-director of the Ministry of Interior's department for research, also feels that compensation should be restricted to applied research, and that steps should be taken to preserve the country's fundamental research base. "Otherwise we could not contribute to European science in the long run," he says. **Oliver Klaffke**

Election costs hit Pretoria science budget

Cape Town. South Africa is not spending enough on research and development, according to the country's new Minister for Arts, Culture, Science and Technology, Ben Ngubane, speaking in his first public address at the Human Sciences Research Council in Pretoria last week.

Ironically, however, Ngubane's speech, in which he committed himself to working towards "more focused and effective science and technology", came only days after the country's three largest science councils had been informed of cuts in their parliamentary grants for the current financial year.

These cuts are part of an across-the-board reduction of 5 per cent of this year's budget for all government departments, rather than a reflection of any deliberate effort to reduce the science budget. Furthermore the overall cut was imposed not by the new cabinet, but by its predecessor,

in order to finance a R1-billion (\$272 million) bill for the recent election.

The reduction has had different consequences for the various research councils because the science vote, despite being voted on as a single budget item, is allocated to five 'mother' departments as funds earmarked for the councils. Departments have been allowed to exercise discretion on where the cuts are applied.

The Foundation for Research Development's R16 million allocation for new equipment in the universities has been cut to R6 million. The largest council, the Council for Scientific and Industrial Research, has been notified of a 5.5 per cent cut to its budget, and the Agricultural Research Council will have its budget reduced by slightly less than this. The three smaller councils — including the Medical Research Council — appear to have escaped the knife. **Michael Cherry**

AAAS criticized over AIDS sceptics' meeting

Washington. The American Association for the Advancement of Science (AAAS) has come under fire from US AIDS researchers and public health officials for its sponsorship of a meeting in San Francisco next month at which speakers will dispute the link between HIV and AIDS.

The meeting's title, "The role of HIV in AIDS: why there is still a controversy", is very similar to that being held by the *Sunday Times* in London next week (see below). It is due to take place on 21 June as part of the annual meeting of the Pacific Division of the AAAS. Speakers include Peter Duesberg of the University of California, Berkeley, the chemist Kary Mullis and nine others. But as criticism of the line-up mounted, AAAS executive officer Richard Nicholson

indicated that the session might be called off. "All options are still open, including cancellation," Nicholson said on Monday.

Critics of the meeting say that nearly all the speakers, who include both scientists and journalists, are known to share Duesberg's scepticism about the role of HIV in AIDS. "This is a real fringe of people surrounding Peter Duesberg who have been saying these things for a while now," says Bernie Fields, professor of microbiology at Harvard Medical School. "AAAS sponsorship makes it sound like a real issue when it's not; I think it's a disgrace."

David Baltimore of Rockefeller University, New York, says he cannot understand why the AAAS has allowed itself to sponsor

a meeting that does not represent scientific opinion. "This is a group of people who have denied the scientific facts," says Baltimore. "There is no question at all that HIV is the cause of AIDS. Anyone who gets up publicly and says the opposite is encouraging people to risk their lives."

The meeting was organized by the AAAS Pacific Division in California, and had not come to the attention of senior AAAS officials in Washington until last week. Nicholson says that the Pacific Division will now "get some new advice" on the proposed session, and "look at it again from scratch."

The executive director of the AAAS Pacific Division, Alan Leviton, says that efforts are now being made to balance the panel. Since the initial line-up was published in a newsletter on 25 April, he has attracted one extra speaker — Gerald Lowenstein of the University of California Medical School at San Francisco — and hopes to confirm two more this week. "I think we're well on our way to getting the additional people we'd feel more comfortable with," he says.

But the session organizer, Charles Geshekter of the California State University at Chico, says that balance is not his priority. "My responsibility is to create an interesting panel and a symposium that will boost attendance at our conference, and that's exactly what I'm hoping to do," he says. "My point wasn't so much balance, it was to generate controversy."

Geshekter is an economic historian of Africa who will present a talk at the meeting entitled "The myth of African sexual promiscuity and misdiagnoses of AIDS in Africa". He says that he organized the meeting in reaction to uninspiring and badly attended AIDS sessions at the main AAAS annual meeting in San Francisco in February. "The argument has been dominated for ten years by one point of view," he says. "It is time to have a more robust debate."

Geshekter says he is "very heartened" by a recent article by Fields (see *Nature* 369, 95; 1994) calling for new approaches to AIDS research. But Fields fears he is being misrepresented by the Duesberg school. "I'm not saying that there is a controversy [about HIV causing AIDS]," he says. "I'm saying that we need to broaden our understanding of the disease. I'd be very worried if my paper was playing a role in giving new life to this [Duesberg] view."

Michael Ascher, of the California Department of Health Services, and Warren Winkelstein, of the University of California at Berkeley, have written to the AAAS journal *Science* questioning the AAAS sponsorship because "some of the views to be expressed ... have potentially serious adverse public health consequences".

Colin Macilwain

... as UK prepares for Mullis encounter

London. Britain's *Sunday Times* has called in Kary Mullis (right), the Californian scientist who invented the polymerase chain reaction (PCR) technique, to back its claim that critics of the theory that HIV causes AIDS have not been given a fair hearing in the scientific press.

Mullis is one of a group of scientists, based in California, who argue that there is insufficient evidence to identify HIV as the 'cause' of AIDS. The newspaper has organized a public meeting in London next week at which Mullis has said that he will mount a broad attack on the way in which much scientific research is managed.

Using as an example not only AIDS but also topics such as global warming, he is expected to argue that many researchers overdramatize the importance of various social problems in order to obtain research grants and further their academic careers. According to Mullis, these have become incentives for researchers to make "false assumptions" about such topics.

But the meeting comes at a time of growing concern among those actively engaged in caring for AIDS patients, both in developed and developing countries, that the *Sunday Times*'s campaign, which draws its legitimacy from Mullis and others, is undermining public and political support for their efforts.

"The paper's campaign has been highly detrimental to AIDS work in this and other countries," says Nick Partridge, chief executive officer of the Terrence Higgins Trust in London, which provides support to AIDS patients. "The most serious impact of the campaign is that it allows those who wish to deny the impact of HIV [as a cause of AIDS] a way of doing so."

Similarly Sue Lucas, who coordinates the AIDS relief work of a number of British charities working in developing countries,

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REASONS

says that there is growing concern among non-governmental organizations in these countries that the *Sunday Times*'s campaign "is encouraging people to think that there is not a problem".

Such concern has been fanned by reports that the British newspaper's coverage is beginning to influence public opinion in countries as far apart as Hong Kong and South Africa.

Supporters of the anti-HIV hypothesis claim that attacks on the newspaper can be dismissed, as they are put forward by those belonging to what they describe as the "AIDS establishment".

But Partridge and others claim that the anti-HIV camp has made rational discourse impossible, as it condemns anyone who criticizes their arguments as a member of a pro-HIV conspiracy.

Next week's debate, for which the *Sunday Times* has set an entry ticket at £15 (\$22.50) is entitled "Why there is still an HIV-AIDS controversy".

But many scientists and AIDS workers see it as less of a scientific debate than a misguided effort to emulate the newspaper's pioneering efforts in unveiling scandals such as the thalidomide tragedies in the 1960s.

David Dickson

US panel backs LHC, but seeks extra funds

Washington. High-energy physics needs an extra \$150 million over the three years 1996 to 1998 both to ensure a healthy US domestic programme and to start collaboration with Europe on the proposed Large Hadron Collider (LHC), according to a key advisory panel to the Clinton administration.

But the panel, chaired by Sidney Drell of the Stanford Linear Accelerator Center (SLAC) in California, said in a report published in Washington on Monday that if the extra money cannot be found, the government should axe unspecified elements of the domestic programme in order to proceed with collaboration on the LHC.

The Drell panel — a sub-group of the High Energy Physics Advisory Panel (HEPAP) — was set up by Hazel O'Leary, the Secretary of Energy, to provide advice on how her department's \$650 million-a-year high-energy physics programme should proceed after last year's abandonment of the Superconducting Super Collider (SSC).

Both White House officials and members of Congress have been eagerly awaiting its report before taking up positions on the future of the field.

The panel said that the extra \$150 million is needed to tide the programme over a period in which large construction programmes at Fermilab, Illinois and at SLAC will strain resources. After that, it added, the budget could return to \$650 million a year (adjusted for inflation).

This would provide sufficient funds both to support the domestic programme and to provide a contribution to the LHC, which would total \$400 million by 2003. Drell says this would enable a "lean" US involvement in LHC. "It's not what some proponents would have wished for, but we're trying to be practical," he says.

Under the plan, little money would go to LHC until 1998. But the panel calls for an early and unequivocal statement from the US government endorsing participation in the LHC, to which European governments are expected to commit themselves next month.

Drell says that international collaboration will not work without an endorsement from President Clinton himself, and that it is needed to show young scientists that the government is committed to the field. The panel said that this endorsement should be made even if the extra \$150 million is not forthcoming from Congress, and the budget remains flat.

Drell warned HEPAP on Monday that if that happened "you guys are going to have to sit down and decide what to kill" from the domestic programme. HEPAP members say privately that possible candidates for extinction would include operations at Brookhaven, New York and at SLAC.

The Drell report has received strong backing from HEPAP itself, and is now being

forwarded to O'Leary, while various panel members are urging the wider physics community to unite behind it.

With real cuts of \$135 million over the past three years in high energy physics funding, Drell says that "we have a programme in trouble." The \$150 million, he says, "is one-and-a-half per cent of the cost of the SSC and would get us through the present crisis so that we could build for the future."

Drell also voiced concern about the extra costs which Department of Energy regulations place on the high energy physics programme. One comparison between the National Science Foundation and the department indicated that the latter was paying 7 per cent extra to meet its own regulations.

"Someone has to look at these things because there is a lot of money going out the door," says Drell. Seven per cent of the programme, he points out, is virtually equivalent to the extra \$50 million a year which his report calls for.

In her first reaction to the report, Martha Krebs, the head of energy research at the Department of Energy, said she understood why it had asked for the extra \$150 million. But she added — perhaps ominously — that

"they've also taken a realistic approach to what the field should do if they don't get it."

The report was welcomed by Congressional supporters of physics. The House science subcommittee chaired by Rick Boucher (Democrat, Virginia) held a hearing on Monday at which Boucher and others — including SSC's chief executioner, Sherwood Boehlert (Republican, New York) — expressed their support for both the LHC and the extra \$150 million.

But political reality remains more fraught than the public reception of the Drell report suggests. A tight budget climate makes it highly unlikely that Congress will restore to high-energy physics money which it has only recently cut. If the budget stays level, Congress will be loathe to implement any HEPAP recommendation to run down facilities in California or New York State in order to fund the LHC in Switzerland.

It is in anticipation of such problems that Drell is stressing the need for a clear lead from President Clinton. But so far there has been no sign from the president's Office of Science and Technology Policy that such a lead will be forthcoming.

Colin Macilwain

Tensions surface in Pasteur dispute

Paris. The Institut Pasteur in Paris is investigating the operation of its laboratory of cellular immunology, some two years after an earlier enquiry found evidence of scientific misconduct by a postdoctoral researcher in the laboratory.

The institute says it wants to know more about the "background" to the misconduct, although it insists that the enquiry concerns only the quality of work in the laboratory, and not the misconduct incident.

But the director of the laboratory, Jacques Thèze, claims that pressure for the second enquiry has come from adversaries keen to exploit what he describes as a minor incident in order to discredit him. In particular, some say that Thèze's efforts to reform the Institut Pasteur in Lyons, which he headed between 1990 and 1992, have made him enemies.

The misconduct affair involved a postdoctoral researcher, Dragana Jankovic, who, according to Pasteur documents, fabricated results from gels in a paper on expression of viral oncogenes submitted to the *Proceedings of the National Academy of Sciences* in 1992.

The paper was withdrawn in October 1992 by Thèze and Moshe Yaniv — a co-author and head of the laboratory of viral oncogenes. Yaniv says their suspicions were aroused when a colleague of Jankovic's, Angelita Rebello, became reluctant to sign

the paper, and that she subsequently told them what Jankovic had done.

When Maxime Schwartz, the director of the Institut Pasteur, heard of the allegations, he promptly set up an enquiry. Schwartz says that Jankovic subsequently confessed and "resigned" (Jankovic could not be traced for comment). He also says that the first enquiry found no evidence that Thèze knew of or suspected the misconduct beforehand.

Nevertheless, according to Schwartz, the second enquiry was set up to address the concerns of some researchers at the Pasteur that aspects of the operation of Thèze's laboratory might have been conducive to Jankovic's misconduct.

But "no smoke without fire" has been one reaction to the second investigation into Thèze's laboratory. Moreover, some argue that the institute must accept much of the responsibility for creating this climate of suspicion.

Furthermore, Pasteur documents also show that the first enquiry found that Antonio Coutinho, the head of the immunology department, and Philippe Kourilsky, his deputy and a scientific director of the Lyons-based company Laboratoires Mérieux, both knew of the allegations of misconduct in June 1992, but waited until October before telling Schwartz.

This finding has been kept confidential, and has created further suspicion, this ►

time over possible motives for the delay in notifying Thèze — who with Yaniv had uncovered the alleged misconduct independently — and the institute of the allegations.

Kourilsky defends his action, however, saying that a tearful Rebello only agreed to tell him and Coutinho about the misconduct if they “swore to secrecy”. He adds that they waited until she brought them proof before proceeding. Nonetheless, Pasteur documents describe the delay as “regrettable”, and say that it “inflated” the misconduct incident.

Kourilsky also claims that the first enquiry found “serious malfunctions” in Thèze’s laboratory, and says that he hopes that the new enquiry will “clarify” matters. But the institute has declined to comment on the exact nature of the alleged “malfunctioning” until the second enquiry has been completed.

In a written statement to the institute, Thèze dismisses such claims, and criticizes the first enquiry for giving “too much weight” to “allegations formulated by individuals implicated by the enquiry”.

One allegation by Thèze’s critics is that he failed to oversee his laboratory in Paris properly because of his prolonged absence as director of the Institut Pasteur in Lyons. But a formal evaluation of his laboratory in April 1992 concluded that it was nonetheless running smoothly.

Both François Gros, a former director of the Paris institute, and Jean-Paul Aubert, director of the laboratory of cellular physiology, have said that it was “normal” for Thèze to have delegated the running of his laboratory while working in Lyons.

Aubert has said the institute should have thanked Thèze for taking on the “difficult and risky” job of running the Lyons institute, instead of “making trouble” for him over a “secondary question”.

Some claim that the charges against Thèze are linked to the local resentment generated in Lyons by his efforts to introduce reforms aimed at addressing longstanding criticism that it carried out too much commercial biomedical analysis, and not enough fundamental research.

Thèze was fired from his Lyons post in early 1992 after conflict with Michel Robatel, the president of the board of the Lyons institute, although the Ministry of Research and the General Inspectorate of Social Affairs both later favourably assessed his reforms. The Institut Pasteur in Paris subsequently tried to withdraw the Pasteur label from the institute, and the historically strained relationship between the two institutes deepened.

Thèze is confident that the conclusions of the new enquiry, which will report its findings to Schwartz next month, will exonerate his laboratory. Schwartz says that the Jankovic affair is the first case of scientific misconduct to have been brought to his attention since he became director in 1988.

Declan Butler

International support urged for biotechnology guidelines

London. Government officials in Britain and the Netherlands are leading an attempt to obtain international endorsement of a set of safety guidelines covering the use of genetically engineered organisms and their release into the environment.

Both countries are now looking for a possible institutional home for these guidelines. One leading contender is the United Nations Environment Programme (UNEP), which already promulgates similar guidelines covering the environmental impact of new chemicals.

The initiative has raised concern among non-governmental organizations (NGOs) such as the Green Alliance and the World Wide Fund for Nature. They are keen that any such guidelines should be backed by a legal instrument, such as a legally enforceable protocol to the Biodiversity Convention.

The need for an international agreement on biosafety was agreed as part of a plan of action approved by the 1992 UN Conference on the Environment and Development in Rio de Janeiro. “From an NGO position, we cannot accept anything that might sabotage an eventual legal instrument,” says Julie Hill, director of the Green Alliance in London. “Guidelines will be useful, but they should eventually become legally binding.”

But British and Dutch officials say that, although they share this goal, agreement on a protocol could take ten years or more. It could also run into opposition in principle from developed countries (such as the United States) committed to deregulation.

International endorsement of a common set of technical guidelines would, they claim, be an interim step. “We do not want to present our proposal as an alternative [to any legally binding instrument],” says one British official. “We want to present it as a set of guidelines that people can pick up and introduce straight away.”

The draft guidelines have been drawn up by an international group of experts. They build both on domestic experience in the developed countries — particularly Britain and the Netherlands — and on feedback from efforts to stimulate regional debates in a number of developing countries on safety in biotechnology.

As they stand, the proposed guidelines deal with topics such as the assessment and management of risks, establishing national and/or regional focal points responsible for the transfer of information about novel organisms, and helping developing countries to strengthen their own expertise in imposing and evaluating safety procedures.

“The guidelines do not waste time trying

to find the perfect solution,” says one British official. “It is a ‘warts and all’ approach, but we hope that it is both a pragmatic and a scientific approach, allowing countries to make the best use of information available at any one time.”

Those responsible for drafting the guidelines say they are intended to meet the

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

A Nigerian gene bank: safety first.

concerns of less-developed countries keen, for example, to introduce genetically engineered crops, but uncertain over how to tackle the safety aspects.

At the same time, they say, biotechnology companies in developed countries are equally eager to see an international safety system in place, if only to reduce the likelihood of embarrassing situations that could give the whole industry a bad name.

The proposed guidelines refrain either from addressing the socio-economic impact of biotechnology on countries at different levels of development, or from defining the precise mechanisms for public involvement in the regulatory process. The guidelines also allow individual countries to introduce more stringent safety requirements.

The main political issue now is which international body is best placed to provide the institutional back-up needed to promulgate the guidelines, and in particular to channel to less-developed countries the resources required to implement them.

The development of the proposed guidelines has the backing of the British and Dutch governments. They are now being circulated widely to officials in other countries, and are expected to be discussed in the corridors at a meeting of the Commission of Sustainable Development to be held in New York next week.

The attitude of the NGOs will be important in winning political approval. These remain adamant that a legally binding international commitment will be needed to protect the environment against novel organisms. But most seem prepared to be pragmatic, and to back the proposed moves as an interim measure that is better than nothing.

David Dickson

Tsukuba bucks tradition to boost academic-industry ties

Tokyo. Tsukuba University, based in Tsukuba Science City north of Tokyo, is flying in the face of Japanese university tradition by taking a bold initiative to promote links with government research laboratories and company research institutes in the science city.

Next month, the university will start recruiting scientists worldwide for a new research organization called the Tsukuba Advanced Research Alliance (TARA). TARA will have more than 200 faculty members, most of them on temporary contracts, and aims to cultivate interactions between government, industry and academic institutions.

The new body will focus on nanotechnology, biological sciences — such as gene regulation, cell-cell interactions and neural functions — multimedia and information management, research management and technology transfer.

It is the first such university-based organization in Japan. With nearly 50 government research institutes and more than 200 private-sector research institutes in Tsukuba, the opportunity seems "obvious", says Tsukuba University's president Leo Esaki, who won the Nobel prize for physics in 1973 and came to Tsukuba in 1992 from IBM.

Last year, the university established a



Esaki: opportunity was 'obvious'.

"study centre" to bring together researchers from public and private research organizations to hold regular symposia and seminars on selected research topics. Out of this emerged the TARA concept which has won substantial funding from the Ministry of Education, Science and Culture. In the budget for fiscal year 1994, which is expected to be passed by the Diet in June, ¥3.6 billion (US\$34 million) has been set aside for a central building, equipment and facilities.

Annual running costs are estimated at ¥4.8 billion yen. A substantial proportion of this will come from private sector sources and government agencies other than the education ministry — highly unusual for a Japanese university.

According to Esaki, many companies, including electronics companies, and pharmaceutical and chemical manufacturers are keen to join TARA to participate in contract and joint research. Esaki expects to raise about ¥110 million a year in research funds from such companies and other government organizations, such as the Ministry of International Trade and Industry and the Science and Technology Agency.

One unusual feature of TARA is that about half the researchers will be recruited from outside the university. TARA will also be able to offer permanent contracts to foreign scientists, following recent changes in university regulations.

Projects will be subject to regular external review by outside scientists, including non-Japanese, again a rare occurrence in the Japanese university and government research system.

David Swinbanks

Concern grows over reaction to deaths in clinical trials

Washington. The deaths of five out of 24 patients taking part in a National Institutes of Health (NIH) drug trial was preceded by a catalogue of errors on the part of the researchers and the sponsoring drug company, according to the Food and Drug Administration (FDA).

But the FDA, having sent warning letters to researchers and companies involved in last year's trial of the drug fialuridine for the treatment of hepatitis B, plans no direct sanctions, provided changes are made to avoid repetition of the errors.

Angry public health groups and congressmen, led by Edolphus Towns (Democrat, New York), chair of the House of Representatives government operations subcommittee, are complaining that agencies are closing ranks to defend the conduct of the trial. In a statement, Towns described the FDA action as "nothing more than a slap on the wrist", and promised summer hearings on the case.

An interim investigation at NIH last summer found that the trial had conformed to NIH procedures. But a more thorough investigation by an outside panel — chaired by David Challoner, vice-president of health affairs at the University of Florida — will deliver a more critical report to Harold Varmus, director of NIH, this week. Donna Shalala, the Secretary of Health, has asked the Institute of Medicine to follow that through with its own independent study.

When the patients died, the initial response of the agencies suggested there had been a tragic but unavoidable accident (see *Nature* 364, 275; 1993). But the FDA letters paint a darker picture of researchers and drug makers turning a blind eye to previously known problems with fialuridine.

The letter to the principal investigator on the trial, Jay Hoofnagle of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), accuses him of failing to give trial participants relevant information about fialuridine in informed consent documents, and of failing to respond appropriately when side-effects arose.

In addition, the drug's manufacturer, Eli Lilly of Indianapolis, is accused of failing to oversee the trial adequately. Revised trial guidelines prepared and implemented by the FDA since the deaths give the manufacturer more explicit responsibility for overseeing trials and, in particular, for ensuring a better flow of information about side-effects from separate trials of the same drug.

Both Hoofnagle and Eli Lilly must respond to these accusations by the end of the month. Sanctions are threatened unless they act to prevent something similar happening again.

Colin Macilwain

Gene-altered tomatoes face new protests

San Francisco. Consumer groups have responded rapidly to last week's official approval of the first genetically engineered whole food, the 'Flavr-Savr' tomato, by preparing a boycott and public 'tomato squashes' to demand labelling of biotech foods.

"We're going to ask every supermarket chain not to stock this tomato," said Jeremy Rifkin, the anti-biotechnology activist. "Otherwise they'll have to label every tomato." The Union of Concerned Scientists has issued a statement describing the government's oversight efforts as "inadequate and potentially dangerous".

On 18 May, Calgene Inc. of Davis, California, won permission from the Food and Drug Administration (FDA) to begin selling the gene-altered tomato which can be left to ripen on the vine, and lasts longer in shipping and on grocery store

shelves. The FDA did not ask Calgene to label its product, and has said it will not require any special labelling of genetically engineered foods.

Calgene volunteered for regulatory scrutiny to ease consumer concern about safety. It has also said it planned to mark each tomato with a "genetically engineered" label. But last week, the company said it would test a variety of labels, including some that did not mention the biotechnology process.

The company says that the tomatoes will be on sale in Midwestern states and California by early June. But several California grocery stores said they would wait and see how customers responded. They also said they were not selling milk from cows treated with bovine growth hormone, another controversial genetically engineered product. Sally Lehrman

Cautious welcome to NIH peer review reforms

Washington. Encouraged by preliminary results, the US National Institutes of Health (NIH) are to expand an experiment in modifying the peer-review system for research grants. The goal is to reduce the time spent dealing with applications that are clearly not likely to win a grant, and to increase that spent evaluating borderline cases.

Nearly 80 per cent of the reviewers who took part in an initial small trial said that they had the same level of confidence in their recommendations as under the current system. Almost 60 per cent said they would approve the new approach if some additional modifications were made.

The approach is known as 'triage', after the military procedure for sorting battlefield casualties into priorities for treatment. Jerome Green, director of NIH's Division for Research Grants (DRG), says that a decision on whether to extend it to the whole of NIH is likely to be made at the end of the year.

There are several reasons behind NIH's desire to modify its peer review procedures. One is to speed up the process; at present, grant applicants have to wait nine months to hear whether they have been successful, and most are disappointed. The NIH can fund fewer than a quarter of the applications it

ited proposals either to a study section within the division, or to a panel within an individual institute, each made up of 15 to 20 senior scientists or specialists in the field.

Before the panel or section meets, the NIH scientist responsible for its work sends every application to two reviewers. These assign a score to the application ranging from 500 (lowest) to 100 (highest), based on factors such as its significance, the appropriateness of methodology, the qualifications of investigators and the resources available in the investigators' home institution.

The scores are passed to the DRG, where they are adjusted to compensate for differences between study section members. The panel then discusses all applications, eventually deciding not to recommend some for further consideration. An NIH staff member then sends unsuccessful applicants a summary of reviewers' criticisms and remarks emerging from the panel discussion.

The applications that survive their scientific peers are sent to the second level of review within the individual institutes, and eventually about 20 per cent of the applications are funded. In general, successful applications have both the lowest scores from the scientific reviewers and are ranked above the fourteenth or fifteenth percentile or so of the overall range of scores.

Triage aims to identify as 'noncompetitive' applications below the fiftieth percentile. If both initial reviewers agree with this assessment, and no other scientific reviewer disagrees, the application is not discussed further and the NIH staff member does not write a summary of the reasons for rejection.

The applicant does, however, receive the criticisms of the two reviewers, thus maintaining most of the tutorial element of peer review. Of the four (out of over a hundred) study sections involved in the trial so far, only the Human Development and Aging Study Section winnowed out all those below the fiftieth percentile, which in that case constituted 51.1 per cent of applications.

The new approach has met with a generally favourable response. Keith Yamamoto, chair of the department of pharmacology at the University of California, San Francisco, who is organizing a seminar on peer review at the NIH in the autumn, says that "on balance" he is in favour of triage: "It gives more time to discuss the top proposals."

At the same time, says Yamamoto, "there is a real danger that innovative research will be triaged out". This concern stems from a suspicion that reviewers, under the pressure of tight budgets, are becoming increasingly conservative, and now favour proposals backed by significant preliminary data.

"I can't tell you how often I have read a review that says this proposal has the potential to advance the field tremendously, but it is high risk," says Yamamoto. "In fact, I've

written that a few times myself."

Yamamoto believes that, in order to preserve high-risk, high pay-off science, the instructions to reviewers should explicitly require them to consider innovativeness along with the other criteria. "What has made American science so enterprising is the ability of some people to move non-linearly," he says. "We must not cut these people out of the loop."

Others are concerned that eliminating the detailed assessments of applications that unsuccessful applicants receive will remove a valuable service to the scientific community, as they are essentially a free consultation with leading scientists in a field.

Green says he is aware of this, but it needs to be put in perspective. "We need to balance the need to shorten the time that scientists and NIH staff spend on each application against the importance of maintaining the tutorial aspect of peer review," he says.

Although the triage trial is to be expanded, a significant reduction in the time it takes for applicants to hear their fate is likely to come only with a move to electronic filing and processing of grant applications. "At present, we are in the ridiculous position where the researchers will have written up their application on computer, yet we receive them and key them in, then print out numerous hard copies," says Green. Tight funding means that full implementation of electronic processing will probably take at least five years.

Helen Gavaghan

Brussels boost to UK universities

London. The research income received by British universities from the European Commission (EC) in Brussels increased by a hefty 32 per cent last year to reach a total of £76 million — 7.0 per cent of their total research funding — according to figures published last week by the Universities Statistical Record (USR).

In contrast, research grants from private industry rose by a meagre 0.8 per cent, to £121 million. This compares to an increase of 5.3 per cent in the previous year, and is "probably due to the recession", says the USR's *University Statistics 1992-93, Volume 3: Finance*.

Some universities did particularly well in boosting their income from EC sources. The University of Cambridge, for example, which has been protesting strongly about having its domestic funds "capped" by the government in order to maintain support for less productive institutions, saw its EC income almost double, to £3.8 million.

□



receives, and reviewers therefore spend much unpaid time evaluating applications that will be unsuccessful anyway. This has created a sense of frustration in the scientific community, making it difficult for the NIH to recruit reviewers.

Unsolicited proposals are the bedrock of biomedical research in the United States, and account for nearly half the NIH's extramural research funding (\$8.5 billion in the financial year 1993). The triage approach focuses on the first of two stages in the review process, that concerned with a purely scientific review of applications. The second involves evaluating applications that pass the first stage against the research and other priorities of the individual institutes.

At present, the DRG assigns all unsolic-

Received knowledge

SIR — Nomenclature is not always easy or interesting for biologists. We prize our terms, perpetuate private jokes in naming proteins, seek importance through obfuscation. It's hard to get too worked up about such matters; they contribute character to our field. On the other hand, clarity and precision are among the virtuous aims of most of our endeavours, so it seems appropriate to mention that the naming of membrane-spanning proteins is approaching a crisis.

For instance, adrenergic receptors are becoming known as 'adrenoceptors', which seems to reduce the word receptor beyond sense. The literature also reflects a desire to describe groups of membrane receptors by the number of times the proteins traverse the membrane. Thus, we have 'single transmembrane spanning receptors' and 'seven transmembrane spanning receptors'.

Recently, some of our colleagues, tongue-tied by such phrases, have resorted to alternatives. 'Seven transmembrane spanning receptors' have been termed 'serpentine receptors'. While this usage conjures up the overall shape of such proteins, it does not distinguish among membrane proteins that may be equally serpentine but that have other than seven transmembrane spans, it risks confusion with the mineral of the same name ($\text{Mg}_3\text{Si}_2\text{O}_5(\text{OH})_4$) and it might even appear to suggest some devious receptor activity *à la* Satan and Eve of biblical fame. Moreover, I fear that such usage will encourage blatant anthropomorphizing and that we will shortly have elephantine or obese receptors (large aggregates, such as the nicotinic cholinergic receptor) and phallic receptors (those, such as the EGF receptor, that penetrate the bilayer once). Enough.

How about a system that draws on an established logic, such as the numerical prefixes used to describe polygons and polymers, in combination with the word 'span', a single traversal of the membrane? Thus, a seven transmembrane spanning protein would be a 'heptaspan'. Such a system would be more precise than adjectives such as serpentine and would avoid the ambiguity of the awkward phrase seven transmembrane spanning receptors in which it is not clear whether seven refers for the number of transmembrane spans or the number of receptors. I propose that we simply combine common numeric prefixes (mono, di (and do), tri, tetra (or quadra), penta, hexa, octa, nona, deca, and so on) with span to create

descriptors for groups of membrane spanning proteins. A few examples:

No. of spans	Noun (Adjective)	Example
1	Monospan (monospanning)	Many growth factor receptors, such as the EGF receptor
7	Heptaspan (heptaspanning)	G-protein-linked receptors, such as the β -adrenergic receptor
12	Dodecaspan (dodecaspanning)	Adenylyl cyclase

This scheme is based on words that convey precise numeric meaning and that most scientists already know. My experience is that after a few tries dotriacontahectaspan (132 spans) will roll off the tongue as readily as hemisemidemi-quaver.

Laurence L. Brunton

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Credit where due

SIR — I was both intrigued and disturbed to read Bob Ward's letter concerning publication rights of the student (*Nature* 368, 579; 1994). Whether true or not, these comments strike at the heart of the student-supervisor relationship, and perhaps a viewpoint from a different perspective may be worthwhile.

First, I regard it as the responsibility of the supervisor to provide an environment in which the student can learn how to perform research and to think for him or herself, while engaged in a programme of research. It is the responsibility of the student to take advantage of those opportunities.

Second, I suggest that in most scientific disciplines the student-supervisor relationship is based less on conflict and more on teamwork in which the supervisor acts as team leader. Thus, where a research programme is supported by a grant, the team leader (supervisor) will probably have had to provide a detailed proposal of research in which the basic ideas, objectives, methods and likely outcomes of the research are presented. From this perspective, the team leader carries the responsibility for the research programme.

Finally, perhaps students could be permitted to submit the results of their research for publication provided that (1) they have financial support independent of a research proposal submitted by the supervisor, (2) the students define the project, perform and interpret the work and write the papers themselves, (3) the institute at which the students perform the

research, and which has a legal responsibility for these students, sanctions publication and finally (4) the students accept full responsibility for the content of the papers. In the sciences in general, the responsibility for a research programme more commonly rests with the supervisor, and the most direct way of expressing that responsibility is by having that person's name associated with the results of that programme in the open literature.

Terence P. Kee

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SIR — The inclusion of supervisors' names on research publications by graduate students is an issue that has been examined closely in preparing for the next Funding Council's Research Assessment Exercise (RAE) to be undertaken in 1996. The funding councils have now completed their review of the responses to the joint consultation and expect to publish a framework document in June outlining the arrangements planned for the 1996 exercise. I am sure that Ward (and others) will be pleased to see that it is proposed that, for the next RAE, publications by research students can be included even if not co-authored by the supervisor, provided the work can be identified as a genuine outcome of a supervised study.

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Carnot fils

SIR — The review of *The Refrigerator and the Universe* (*Nature* 368, 598; 1994) is almost as baffling as the second law. Could the swashbuckling Carnot (1753–1823) pictured by the founder of thermodynamics? Hardly, for he died a year too early. It is, of course, Lazare Carnot, the 'Organizer of Victory' of the French Revolution, who took time out, in 1795, to beget his illustrious son (it will always be a mystery how this associate of Robespierre and St Just avoided the guillotine, except that he ran the army). As to the author of the second law, curiously described here as Nicolas Carnot: true he was baptised (assuming baptism was practised in Paris in 1796) Nicolas-Léonard Sadi, but he has been universally known as Sadi Carnot (1796–1832). To keep things completely straight, the fourth president of the Third Republic, Marie-François Sadi Carnot (1837–94) was Sadi Carnot's nephew, and Lazare's grandson.

H. R. Catchpole

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Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only.

Bringing more order out of noisiness

Ambitions to detect meaningful signals in the presence of noise go back to the early telegraphs of the nineteenth century, but are now embodied in the search for proofs of what is called stochastic resonance.

THE idea that noise, an essentially random process, may assist in the establishment of order is a strange one, but must now, it appears, be taken seriously. Indeed, there is now even a name for the phenomenon: stochastic resonance. Contradiction in terms though that phrase may seem, it is increasingly to be found scattered through the physics journals, suggesting that a minor industry is in the process of emerging.

The essence of stochastic resonance is that a weak signal can be made more apparent, or even amplified, in the presence of noise, which makes plain the paradoxical character of the phenomenon. Nobody would expect that to happen at a cocktail party, for example. It is also relevant that stochastic noise has nothing to do with the way in which "non-white" noise can be used to win directional motion from a structure like a microtubule or a muscle fibre (see *Nature* **369**, 181; 1994). There, if the noise is not equilibrium or "white" noise, directional motion is incompatible with the second law of thermodynamics. But stochastic resonance works with white noise, and is a distinct phenomenon.

A few simple examples would help, but the simple ones seem all somewhat artificial. But for what it is worth, consider a particle trapped in a double potential well — a structure that will confine the particle to one of two positions separated from each other by a certain minimum energy, say q .

If the system is then immersed in a heat-bath, which is the conventional way of supposing that the particle from time to time has the energy it would have if it were in thermal equilibrium with, say, a perfect gas, there is then a chance that it will switch from one position to the other in any interval of time. And as in brownian motion proper, the chance will increase as the temperature of the heat-bath increases. At the equivalent of a very low temperature, it will hardly ever switch; at high temperature, it will be switching all the time as if q were zero. That much is just common sense.

Now suppose that the particle is also subjected to a periodic force whose tendency is to make the particle jump from one position to the other and back again. If the external temperature is zero and the periodic force is insufficient to carry the particle over q , there will be no jumping. If, by contrast, the temperature is very high, the switching will again be frequent, so frequent as to swamp the effects of the periodic force.

But then it is reasonable to expect that, at some intermediate temperature, the particle

will jump between the two possible positions more or less in time with the external forcing signal. It may miss some cycles of the oscillation, but there will be long-term coherence between the forcing signal and the response of the particle made possible only by the presence of noise.

That hand-waving argument has respectable antecedents. More than half a century ago, H. A. Kramers at Leiden outlined the behaviour of thermally driven particles in a potential well, which has since been widely used in solid-state physics and elsewhere. Even when quantum mechanics is not involved, the problem of a particle jumping between neighbouring potential wells is far from trivial.

If, for example, q is greater than the energy kT , where k is Boltzmann's constant and T is temperature, brownian hops across the barrier will necessarily be rare. But that implies that if a particle makes the jump, the chance that it will lose its energy again will be only small, so that it is likely to keep on oscillating from one well to the other before it settles down. To make the problem meaningful, some kind of energy dissipation is needed. With that proviso, the Kramers result is that the average residence time of the particle in one half of the double well or the other is proportional to $e^{q/kT}$, where the proportionality constant is the relaxation time against the loss of energy.

That behaviour was literally confirmed two years ago by an elegant experiment due to Adam Simon and Albert Libchaber at the NEC Research Institute at Princeton (*Phys. Rev. Lett.* **68**, 3375–3378; 1992). They made video films of the oscillation of $0.1\mu\text{m}$ silica spheres in water between two adjacent potential wells fashioned by focusing two laser beams on spots $1\mu\text{m}$ apart on the cover slip. With the same set-up, they were also able to measure the response of silica spheres to external harmonic forcing (by modulating the relative intensity of the two laser beams). Not surprisingly, they found that the hopping was most nearly synchronous with the external forcing when the frequency was that corresponding to the Kramers frequency (the inverse of the average residence time).

People are not in this business just to confirm what Kramers predicted, however. And there are potentially important fields of application. Indeed, one of the first suggestions that there might be a phenomenon deserving the name stochastic resonance came more than ten years ago from those who build computer models of the Earth's climate, and who were puzzled by the per-

sistence in climate predictions of periodic signals that appeared to be simply artefacts of the starting conditions.

Turning a nuisance into an explanation, there have now been several attempts to relate such phenomena as the repetitiveness (periodicity is too strong a word) of glaciations during the Pleistocene to stochastic resonance. The hard truth is that there is no evident correlation between glaciations and the supposed periodicities of solar forcing of the Earth's climate that Milankovitch says should be brought about by fluctuations of the solar-terrestrial orbital parameters.

Meanwhile, the theoreticians have been busy, at least at the University of Pisa, the University of Missouri and at Georgia Tech (Atlanta). The plan has been to define the conditions under which a weak signal will be amplified into one of its own kind with the help of noise. Since the equations are non-linear, the conclusions have tended to be recounted as the results of simulations.

So have been experimentalists, and in unexpected ways. Last September, for example, four people from the University of Missouri (Douglass, J. K., Wilkens, L., Pantazelou, E. & Moss, F. *Nature* **365**, 337; 1993) described their search for stochastic resonance in the behaviour of crayfish (*Procambarus clarkii*) neurons. Part of the inspiration for the search was the expectation that neurons are adapted to the detection of meaning in the presence of noise. And the investigations showed that firing spikes do appear to occur at intervals related to the periodicity of a forcing potential even when it is virtually swamped by noise.

Kurt Wiesenfeld from Georgia Tech and the Missouri group have now taken the argument further by devising a model neuron that can be represented mathematically (*Phys. Rev. Lett.* **72**, 2125–2129; 1994). Their idea is that a particle (neuron) can move significantly only by surmounting a barrier (become polarized), will then move (fire), but after a fixed time will return to its original state (become depolarized), whereupon the cycle can begin all over again. With the help of a few crayfish neurons and some sophisticated measurements, it appears that the agreement between the predictions of the model and the measurements is better than anybody had reason to expect. The amplifying potential of stochastic resonance may not yet have been turned into a repeater for the telecommunications industry, but the hunt for the phenomenon will plainly be enlivening for us all.

John Maddox

Death gets a brake

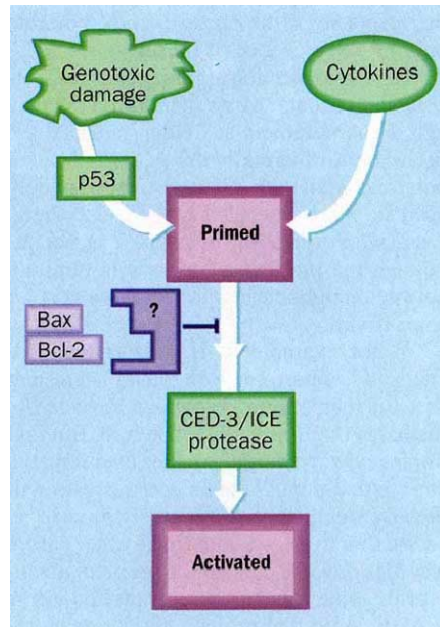
Andrew H. Wyllie

PAPERS by Hengartner and Horvitz¹ and Yin *et al.*² (pages 318 and 321 of this issue) provide fresh evidence for the way in which members of the *bcl-2* gene family interact to preside over the cellular decision between life and death. The decision to die is often made actively by cells, initiating the process of apoptosis in response to local cytokines, signals from viral genes or injury. Hence information on the genes regulating apoptosis bears on a variety of disciplines, including developmental biology, cancer research, immunology and toxicology.

bcl-2 is the prototype of a family of genes that inhibit apoptosis. It was originally cloned from the t(14;18) translocation breakpoint found in follicular B-cell lymphomas. In 1988, Vaux *et al.* showed that *bcl-2* expression rescued lymphoid and myeloid cells from an otherwise inevitable death caused by withdrawal of the cytokine interleukin-3 (ref. 3). The rescued cells did not proliferate, however, showing Bcl-2 protein to be a true 'survival factor' in this situation, distinct in action from mitogens. It soon became clear that *bcl-2* expression has a key part in the physiological process of affinity maturation of B cells in follicle centres, specifying the survival of the small minority destined to become memory cells following antigen challenge.

B-cell selection is only one aspect of the biology of *bcl-2*, as indeed *bcl-2* is only one element in a cascade of genes regulating apoptosis. So how do these genes interact? A powerful method to address this question has been the painstaking analysis of mutants of the nematode *Caenorhabditis elegans* that show abnormal numbers of cell deaths during development⁴. Genes that control events at different points in the death pathway can be ordered relative to each other by study of double mutants. *ced-3* and *ced-4* define the initiation of the killing event. Downstream lie genes encoding effector molecules involved in recognition for phagocytosis; upstream lies *ced-9*, whose activity is required to repress *ced-3* and *ced-4*. The products of both *ced-3* and *ced-9* have mammalian homologues: CED-3 with the protease interleukin-1 β -converting enzyme (ICE)⁵, and CED-9 with Bcl-2 (ref. 6). There are further regulatory genes upstream of *ced-9* in the nematode. Mammalian cells destined to die because of widely dissimilar stimuli can almost all be rescued by expression of *bcl-2* (refs 7, 8), showing that the Bcl-2 protein must act close to the final irreversible step where the various afferent pathways converge and at which the effector processes are activated (see figure).

How does Bcl-2 work? The protein has a carboxy-terminal membrane anchor, and is localized to the outer mitochondrial, nuclear and endoplasmic reticulum membranes⁹. Lipid membranes are sites in which reactive oxygen intermediates (ROI) engage in potentially damaging lipid peroxidation chain reactions. An attractive hypothesis is that Bcl-2 protects



Regulation of apoptosis in mammalian cells. By distinct but convergent pathways, lethal stimuli prime cells for apoptosis. But Bax–Bcl-2 dimers place a brake on its activation, perhaps through an intermediate protein. Once this is released the coordinated effector processes are initiated (for example cell shrinkage, surface changes permitting phagocytosis and endonuclease activation), apparently through the CED-3/ICE-like protease.

cells by inhibiting lipid peroxidation, even in the presence of ROI. Perhaps surprisingly, if this were its sole function, attachment to the membrane is not essential, as *bcl-2* deletion mutants lacking the anchor domain are at least partially effective in protecting cells from death¹⁰.

Yin *et al.*² introduced mutations into the two regions of *bcl-2* where homology with other members of the family is particularly high. Two critical amino acids were identified (Gly 145 and Trp 188) as essential for Bcl-2's function as a survival factor. Mutations involving these amino acids also destroyed the ability of Bcl-2 to heterodimerize with a protein called Bax, although the ability of the mutant Bcl-2 proteins to form homodimers, or dimers with wild-type Bcl-2, was unaffected. Bax is itself a member of the Bcl-2 family; although it also forms homodimers, these

are ineffectual for cell survival — indeed, they accelerate apoptosis¹¹. The conclusion is that the survival factor function of Bcl-2 depends acutely on the stoichiometry of its interaction with Bax, and this in turn depends on the amino-acid sequence in the two conserved dimerization motifs.

Other members of the Bcl-2 family exist: Bcl-x and Mcl-1 are cellular products, and there are also interesting viral homologues, all similar in amino-acid sequence. Moreover, Bcl-2 interacts with other proteins, such as R-Ras. So it may have several alternative partners in the cell, and there might then be many ways in which the critical stoichiometry favouring survival could be disturbed.

Hengartner and Horvitz¹ present new data defining the nucleotide change and functional properties of a gain-of-function mutation in *ced-9*. Interestingly, the mutation is a single Gly-to-Glu substitution in codon 169, the equivalent to codon 145 in human Bcl-2. Although wild-type human Bcl-2 can rescue CED-9-deficient nematode cells, Bcl-2 with exactly the same 145 Gly-to-Glu substitution was ineffective: far from showing gain of function, it rescued neither nematode nor mammalian cells, nor did it form heterodimers with Bax. Because we know nothing of the nematode version of Bax, it is difficult to ascertain the significance of this failure, although it does emphasize the exquisite sensitivity of the relationship between these protein–protein interactions and the life-or-death decision.

Finally, Hengartner and Horvitz show that the gain-of-function *ced-9* mutant is less effective in generating extra surviving cells when it is present as heterozygote with the wild-type as compared with a null allele. This result might be expected if one considers wild-type and gain-of-function proteins to be in competition with each other for the nematode Bax partner. The other explanation suggested by the authors is that two forms of CED-9 protein exist and have opposite effects on cell survival. Both explanations may merge if the situation is akin to that of another member of the vertebrate *bcl-2* family, *bcl-x*. This gene encodes two splice

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variants, one with survival-factor function and one with the opposite effect¹²: presumably they also dimerize with each other or another partner, as they share the two highly conserved Bcl-2 dimerization motifs.

Tantalizing questions remain. What is the immediate downstream target of the Bcl-2–Bax dimer? How does this target activate the CED-3/ICE-like protease? More generally, why should the Bcl-2 brake on death — one of the last default opportunities before apoptosis becomes inevitable — be critically dependent on two components, each of which might be rendered unavailable by binding to other proteins (or other copies of itself)? Perhaps in this way cell sensitivity to apoptosis can be regulated coordinately with the on–off switch that activates the process. Thus a cell with little Bax would require only low levels of Bcl-2 to reach

the critical stoichiometry at which Bcl-2–Bax dimers form, and reprieve from death is assured. Conversely, high Bax levels might characterize a cell that is more sensitive to death and more difficult to rescue. Intriguingly, p53 protein, which is a well-established activator of one of the pathways to mammalian apoptosis, differentially affects the levels of Bax and Bcl-2 (ref. 13). Control of the Bcl-2–Bax brake may hold the clue to the precise location of deaths in development, the differing sensitivity to death of cells of different lineage or of differing hierarchical status within one lineage, and the huge medical problem of drug resistance in cancer chemotherapy. □

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TECHNOLOGY

Devices and desires

Louis Brus and Don Eigler

THERE is a sharp contrast between the current rapid progress in science at the nanometre scale and the longer-term aim of converting newly discovered quantum devices and nanometre materials into useful engineering nanotechnology. That, at least, was the message of a workshop last month*, covering a very broad range of science.

Much of the recent boom in nanoscience derives from the use of scanning tunnelling microscopy (STM), near-field optical microscopy and the like to characterize and, even more remarkably, modify surfaces atom by atom. Spelling out names with adsorbed atoms on a metal surface at low temperature became possible several years ago. Now we are seeing a big step forward: an STM method has been developed for both supplying and chemically bonding hydrogen atoms to single, specified surface silicon atoms on a Si(111) surface with the (7×7) reconstruction at room temperature¹ (M. Aono, RIKEN). In normal operation the STM instrument images (without modifying) the surface hydrogen atoms; yet by applying a voltage pulse over one surface silicon atom, a hydrogen atom adsorbed on the platinum tip of the microscope can be transferred and bonded. This process is important technologically, as hydrogen termination of silicon crystals prevents surface carrier recombination. Here, one can in principle see the beginning of device construction atom by atom. In essence, the STM instrument is a macro-

scopic machine being used to detect and cause the formation of chemical bonds one by one.

An equally striking result is the achievement of near-field scanning optical microscopy in obtaining the luminescence spectra of individual dye molecules, at room temperature in air, essentially under physiological conditions². The 100-nm resolution and superb detection sensitivity of this probe allow a single molecule adsorbed on a polymer surface to be repeatedly excited and detected so that its spectrum can be built up photon by photon. Optical nanoscience, at a resolution less than an optical wavelength, is just beginning to be explored. This, like the silicon hydride experiment, is an example of nanoscience results that were unthinkable just a few years ago.

In the creation of new materials, clusters and devices, there are two approaches: top-down and bottom-up. The top-down approach applies electron beam lithographic techniques, as used in the semiconductor industry, or scanning probe methods, to fabricate nanometre structures designed to explore quantum aspects of electron transport physics. For several years now, electron beam lithography has been used to make 'artificial atoms' — metallic regions containing a precisely counted number of extra electrons at liquid-helium temperature — and these have been formed and designed into single-electron transistors and circuits. Just last year³, the coherent transfer of superconducting Cooper electron pairs in such devices was observed (M. Devoret, CEA Saclay).

These low-temperature, single-electron circuits suggest an ultimate 'quantum computer' where computation would be done coherently and reversibly, without energy dissipation, unlike in present computers. However, the realistic operating principles of a quantum computer are not clear; R. Landauer (IBM Yorktown Heights) argued that present schematic proposals are not practical from several perspectives. For example, any design must allow for some energy dissipation in order to correct accumulating error due to manufacturing irregularities.

In the bottom-up approach of chemistry and materials science, the goal of designed molecular self-assembly is explored in the liquid-phase synthesis of organic 'super-molecules' and semiconductor nanocrystals. In molecular rotaxanes, bead-like rings shuttle along an organic chain from one aromatic docking station to another, in something like a nanometre abacus (J. F. Stoddart, Univ. of Birmingham).

To be useful, however, such molecules must be assembled and controlled. Now, they have been crystallized into an organized layered structure⁴. Electrochemical and photochemical schemes for controlling bead position in such machine-like devices have been devised (Stoddart; see also ref. 5). These steps in the chemistry of designed self-assembly, part of a growing worldwide effort, support Stoddart's vision that "chemistry is actually a young science".

Can we use these new devices? The most advanced effort is the miniaturization of sensors, actuators and machines to the micrometre rather than the nanometre scale, for direct combination with today's silicon integrated circuits. In fact, silicon micromachined mechanical resonators are already being used in Rolls–Royce engines (H. Guckel, Univ. of Wisconsin, Madison), and amperometric biosensors for glucose are directly integrated with silicon circuits (C. R. Lowe, Univ. of Cambridge).

However, on the nanometre scale we simply do not have a robust, practical method for mass production. Electron beam lithography and scanning probe machines are too slow, in that they make devices one by one, instead of in parallel as in optical lithography (A. Broers, Univ. of Cambridge). In present computers there were two outstanding inventions: the individual transistor and mass manufacturing of integrated circuits. Nanotechnology is now in the single device invention stage, and there is no clear vision of how one could practically

*NATO Advanced Research Workshop on *The Ultimate Limits of Fabrication and Measurement*, Cambridge, UK, 5–8 April 1994.

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integrate devices in a second stage.

A search for new functionalities and new types of processes, and not just improvements in known devices, might provide the innovative thinking required. There is an increasing, worldwide effort. At the National Institute of Advanced Interdisciplinary Research in Japan, for instance, the Atom Technology Project has its sights set on the ultimate manipulation of atoms and molecules, as described above in hydrogen bonding to silicon surfaces. Somehow, industrial manufac-

turing and synthetic chemistry will merge on the nanometre scale; this is the idea behind designed self-assembly. As H. Rohrer of IBM Zürich put it in the final discussion, we should remember that "tomorrow is not an extrapolation of today". □

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INTERSTELLAR MEDIUM

Fullerene's faint fingerprint?

Harold Kroto

HISTORY has shown that astronomical observations can lead to some of the greatest advances in scientific understanding, from Newton's discovery of the colours of the Sun's spectrum, through the discovery of helium in the Sun to our understanding of the origin of the chemical elements as the products of nucleosynthesis in stars. Today, few more fascinating puzzles exist than the identities of the carriers of the diffuse interstellar bands (DIBs), a set of absorption bands seen in the spectra of stars observed through dusty regions of the Galaxy. On page 296 of this issue, Foing and Ehrenfreund¹ point to a possible culprit.

For sixty or so years it has been recognized that the DIBs are not associated with the stars themselves but with tenuous material in the space between the stars — the interstellar medium (ISM). Herbig² carried out a definitive and valuable overview of the characteristics of these features in 1975; since then the number of known DIBs has quadrupled from about 50 to about 200, largely because of advances in astronomical instrumentation.

The features are almost certainly caused by electronic transitions, but they are too broad to be due to free atoms. In recent years the consensus has been (arguably) that the carriers are moderate to large molecules, containing at least 6–10 atoms, where the breadth is due to unresolved fine (rotational) structure. But other suggestions, for example that they are small molecules whose spectral lines are broadened by predissociation, or atomic ions trapped in solid ice or other particles where the broadening is caused by interactions between the ions and the lattice, still cannot be ruled out completely.

Recently, however, the combination of advances in astrophysical spectroscopy, ingenious technical developments in the laboratory and some serendipitous discoveries has offered hope that the identities of these shadowy characters which

lurk in the dusty highways and byways of the Milky Way may be on the verge of being revealed. Over the years countless guesses have been made, but a handful of laboratory and astronomical observations that are truly worthy of the problem have at last appeared.

For one, Krelowski and Walker³ have obtained important 'family' correlations among the stronger features and thus shown unequivocally that there must be several carriers. Another considerable advance came when Sarre⁴ and Fossey⁵ recognized that one of the sets of related features was observable in emission (rather than absorption) from a planetary nebula. The most elegant and convincing experimental contribution to the whole story so far came from Maier's group last year⁶: they provided compelling evidence that some of the DIB features are due to highly unsaturated carbon chain molecules, C_nH_m , with $n = 6, 8 \dots$ and $m = 1, 2$ (that is, molecules such as $C=C=C=C=CH_2$). Now, Foing and Ehrenfreund publish data on some new DIB features that they have discovered, which are tantalizingly consistent with laboratory measurements on the buckminsterfullerene positive ion, C_{60}^+ .

These studies^{1,3–6} stand apart from many previous contributions because in addition to containing reliable data they satisfy, perhaps for the first time in the long-drawn-out history of the DIBs, the essential criteria for good science: the results provide a clear basis for further investigation, and the proposed explanations are susceptible to verification or falsification by techniques that are accessible now — or nearly so.

In 1985, when C_{60} was first discovered, an immediate conjecture was that as the species was produced spontaneously under the same conditions as carbon chains and carbon soot — both known to be associated with the DIBs — it might in some way be responsible for some of the DIBs⁷. Indeed, one of the principal mo-

tives of the experiments that uncovered the existence of C_{60} (described in detail in ref. 8) was to probe the hypothesis made by Douglas⁹ in 1977 that carbon chains might explain some of the DIBs. Ever since Klemperer's ingenious hunch¹⁰ that a particular unidentified radio line might be the ion HCO^+ , it has been clear that complex molecular ions are very abundant in the ISM and likely to give rise to prominent interstellar spectra. Such arguments led to the original proposal that the ion C_{60}^+ might be more abundant than the neutral species in the ISM¹¹ and to suggestions that other C_{60} analogues might be important in space¹².

The successful 'mass production' of C_{60} by Krätschmer and co-workers¹³ was the key step that enabled laboratory spectra of the ion to be obtained by the groups of Schatz¹⁴ and Maier¹⁵. Foing and Ehrenfreund have carefully searched the regions of astronomical spectra where these laboratory data suggest C_{60}^+ features should lie and have found bands which lie within the expected range. A note of caution is warranted, however: the spectra have, as yet, only been obtained in cold argon and neon matrices and are thus subject to matrix shifts from the gas-phase frequencies.

These new observations are undeniably exciting, although the final, definitive identification can only be made by fitting the astronomical features to those of the cold, isolated molecule. Nevertheless, Foing and Ehrenfreund have made a considerable step forward in carrying out this careful observation and analysis programme, and present a well argued case for the existence of C_{60}^+ in space. They clearly identify the final step: it is the measurement of gas-phase ion spectra in the laboratory. A formidable challenge, but not an impossible one, given the tremendous advances in molecular ion spectroscopy in recent years. I have every hope that very soon we will be able to say that C_{60} is indeed a "celestial sphere"⁸. □

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Time for Boxgrove man

Clive Gamble

HALF a million years ago there was no need for a Channel tunnel. Britain was a peninsula to which now-extinct species of rhinos, bears and voles migrated, and these were only part of a rich animal community that clearly lived in a temperate climate. Humans were part of that community, but until now their presence has been known only from stone artefacts. This changes with the discovery, described by Roberts *et al.* on page 311 of this issue¹, of a human leg bone at Boxgrove, West Sussex, which dates to 524,000–478,000 years ago.

Boxgrove has an international significance beyond providing evidence of the oldest Englishman (the sturdiness of the tibia almost certainly indicates a male). The find highlights the status of the site as one of the most important localities for studying hominid behaviour during the Middle Pleistocene (730,000–130,000 years ago). The tibia will attract wide attention. But it should not overshadow the patient, interdisciplinary fieldwork at the site that has been carried out for almost ten years now, with the support of English Heritage. Such long-term funding would never have happened if the site had not consistently produced high-quality archaeological data.

I remember my first visit to the excavations in 1985, and the realization that

the small test pits were revealing an archaeological landscape rather than a single, small site. The degree of preservation was shown by the butchered animal bones associated with stone tools, which had been knapped on the spot from flint grubbed from the collapsed sea cliff and then left behind. It was even possible to see where the human inhabitants had knelt to knap the handaxes. In the laboratory, the business of fitting the stone flakes back together again has provided clues to the operational intelligence that was involved in making the tools. The Boxgrove landscape vividly records a series of brief activities, maybe as little as 15 minutes each, that took place 500,000 years ago on a beach in southern England.

But how can one leg bone contribute to a better understanding of the archaeology of the earliest Europeans? For one thing, the bone will focus attention on the chronology of this and other sites. There are no reliable absolute dates for Boxgrove but there is something just as good — the vole clock. Palaeontologists working on Pleistocene sites in Europe have charted a signal evolutionary change in watervoles from the early *Mimomys savini*, whose molars have roots, to the extant *Arvicola terrestris*, whose molars do not^{2,3}. Half a million years ago the clock struck across Europe to mark the transition in this continuum. The watervoles from Boxgrove (*Arvicola terrestris cantiana*) date to a period shortly after the transition. When combined with the British sequence of faunal changes, brought about by the massive Anglian ice advance that occurred after the Boxgrove temperate phase, it is apparent on biostratigraphic evidence that the site dates to marine isotope stage 13 (524,000 to 478,000 years ago)⁴.

The cross-checking possible at Boxgrove is the key to its chronological importance. Contrast it with claims for very early ages, 1.8–1.6 million years, of poorly

provenanced hominid skulls from Java⁵. The dates for this material have to be taken at face value because they cannot be cross-checked with any closely associated biostratigraphic data. Despite the use of absolute dating techniques, the results are of lesser chronological value than the age estimates provided by the vole clock and its supporting evidence.

What, then, do we learn by getting the

Quality of evidence for human occupation of Europe	
Before 500,000 years ago	After 500,000 years ago
No human remains.	Human remains common.
Contested 'primitive' chopping tools and flakes.	Uncontested stone tools, Acheulean handaxes.
Very small artefact collections selected from a natural pebble background; few or no stone refits, suggesting natural fracture.	Large collections of artefacts; excavated knapping floors with refitted stone nodules, indicating human manufacture.
'Artefacts' found in coarse matrix, disturbed contexts.	Artefacts found in fine-grained matrix, primary contexts.

age right? In November 1993 a workshop held under the auspices of the European Science Foundation examined the claims for the earliest occupation of Europe⁶. Using the vole clock, a notable early site with good archaeology (Isernia in central Italy) has had to be re-dated from 730,000 years old, originally established by absolute dates and palaeomagnetism⁷, to soon after the transition to *Arvicola* 500,000 years ago. The case for even older archaeological sites⁸ of up to two million years old in southern France, Spain and Czechoslovakia looks weaker still: there are no widely accepted hominid remains and the human origin of the stone artefacts is disputed⁹. Indeed, as W. Roebroeks and T. van Kolfschoten of the Rijks Universiteit Leiden pointed out at the meeting, the archaeological record either side of 500,000 years ago is so different that it strongly suggests that humans were not present in Europe before this date (see table). The discovery of



Digging deep — a view of the Boxgrove site.

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a hominid at Boxgrove is another piece in the pattern, comparable to later hominid finds and archaeology at Swanscombe (England), Bilzingsleben and Ehringsdorf (Germany), Arago and Biache (France), Atapuerca (Spain) and Vertesszöllös (Hungary).

The quality of the Boxgrove and European data now sets the agenda for what promises to be a crucial debate in early human prehistory as the implications of a late colonization are explored. Should we now be equally critical of the evidence that supports claims that the earliest migration out of Africa began at least one million years ago? But if greater ages are accepted

for remains in other parts of the Old World — for example the *Homo erectus*-like mandible, excavated at Dmanisi in Georgia, dated at more than 1.4 million years old¹⁰ — how do we explain the presence of humans at the gates of Europe for almost a million years before colonization took place? We are on the verge of an intense, worldwide investigation of early humans as colonizing species¹¹ and of the contribution this aspect made to the evolution of human behaviour. □

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SYNTHETIC CHEMISTRY

Not through the usual channels

Luis Echegoyen

LAST year, researchers at the Scripps Research Institute reported the self-assembly of cyclic octapeptides to form regular nanotubes¹. On page 301 of this issue, Ghadiri and co-workers extend their work and show that these beautiful nanotubes can also self-assemble inside lipid membranes to function as efficient cation transporting channels². The proposed ion channels conduct sodium and potassium at a rate that is almost three times that observed for gramicidin A, a natural pentadecapeptide which is able to form a dimeric helical channel structure that spans the thickness of a membrane. As a cation transporting channel, gramicidin A is very efficient, and the fact that a

synthetically produced compound can rival its properties is in itself remarkable. The additional fact that the channel structure self-assembles from relatively simple individual units is nothing short of spectacular. Overall the strategy is powerful and versatile, yet simple.

Facilitated ion transport across cellular membranes can occur by one of three basic mechanisms: directly through a channel or pore structure in the membrane, via complexation with a carrier, as in a shuttle, or via a relay of closely spaced sites. These processes are shown schematically in Fig. 1. By far the most efficient of the three is the channel, as it does not involve diffusion of a complex or

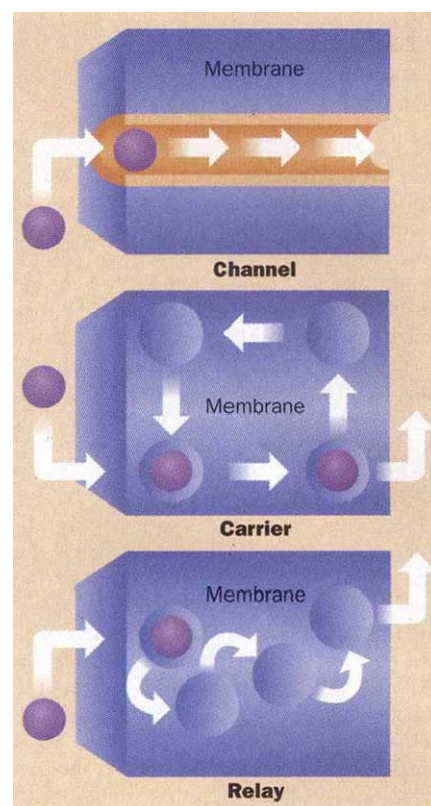


FIG. 1 Three basic ways of transporting ions across a membrane.

cation percolation through the membrane medium. Not surprisingly, this is the one found in natural transporting systems, such as the protein aggregates that form selective ion channels³.

Although structural information about these complex protein aggregates is beginning to come together, most of our

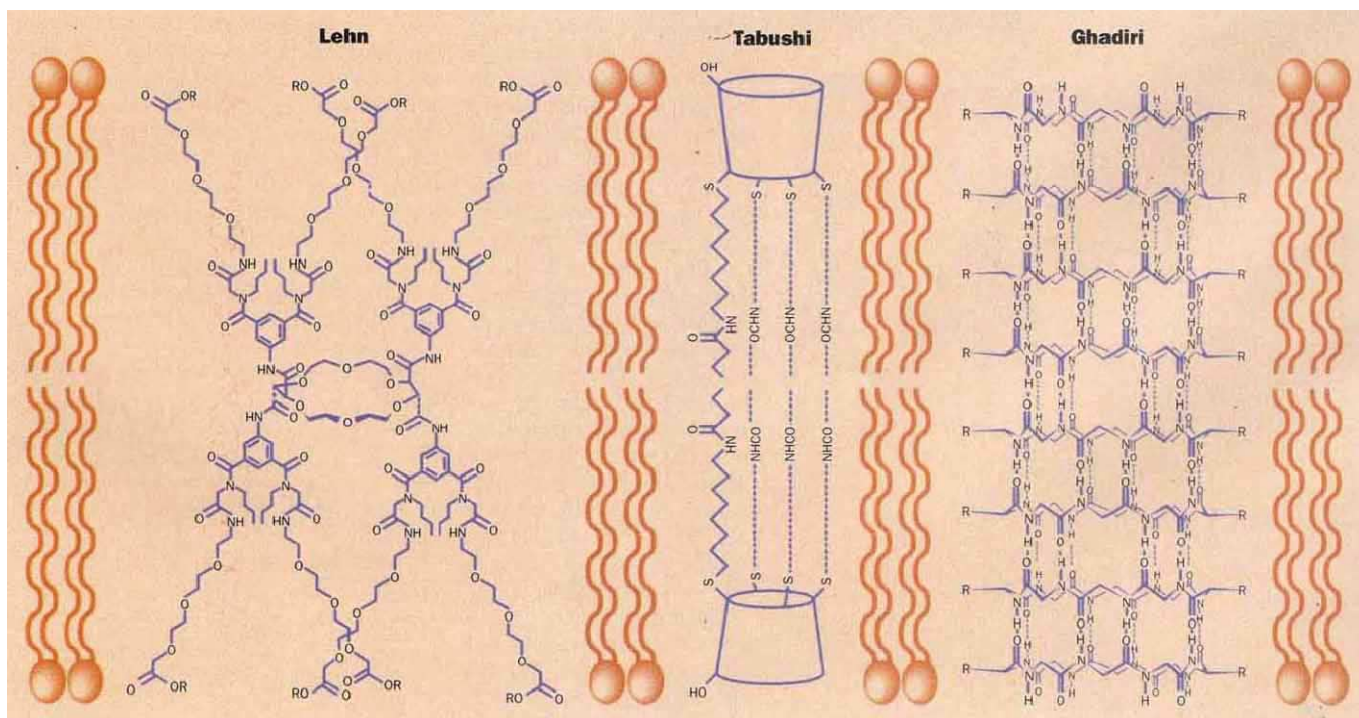


FIG. 2 Synthetic ion channels developed by Lehn's group⁵, Tabushi *et al.*⁷ and Ghadiri *et al.*¹ (page 301 of this issue).

knowledge of ion transport across membranes comes from the study of lower-mass polypeptide ionophores such as gramicidin A (ref. 4). Based on these, many synthetic structures that mimic the function of ion channels have been dreamed up, and some have been prepared and characterized⁵⁻¹⁰. The hope is that artificial channels might allow control of the flow of ions (and molecules) across biological membranes, and be pharmacologically useful. Some of the channels prepared so far do indeed show reasonable ionic conductivities in membranes, but most involve complicated and cumbersome synthetic strategies and, in many cases, their effectiveness is questionable. So Ghadiri and colleagues' far simpler method stands out from earlier work. The presumed structure of the channel formed in their synthesis is represented schematically in Fig. 2, along with two earlier synthetic channel systems.

The secret behind Ghadiri's design is the use of an even number of alternating D- and L-amino acids which can adopt a flat ring conformation. These rings can then interact with each other via multiple hydrogen bonds to form a self-assembled cylindrical β -sheet architecture such as the one shown in Fig. 2. Perhaps more important, appropriate choice of the amino-acid residues allows control of the surface properties of the assembled nanotubes, an important factor in getting them to be incorporated into the membrane.

The cyclic peptide that they have designed and prepared is cyclo[-(Trp-D-Leu)₃Gln-D-Leu-], in which the single L-glutamine (Gln) is introduced to simplify the preparation. The hydrophilic pore diameter of this cyclic structure is 7.5 Å. There are two main driving forces to form the desired stacks: the most obvious is the formation of the multiple intramolecular hydrogen bonds (enthalpic), but there is also the interaction between side chains and lipids (entropic). The latter provides fine control to regulate the incorporation of the individual units into the membrane environments.

For comparison, the authors also look at two model cyclic octapeptides. In one of these, intrastack hydrogen bonding is possible, but the residues are not

sufficiently hydrophobic. The other has the right hydrophobicity but lacks the ability to form hydrogen-bonded stacks. Neither of these was effective for cation transport, so the authors can safely conclude that both driving forces for the self-assembly process are needed to obtain ionic conductivities.

To complete a channel structure that spans the average thickness of a membrane bilayer, about eight to ten units are needed in each stack, assuming an average intersubunit distance of about 4.7–5.0 Å. If the drawing in Fig. 2 reflects the true structure of the synthetic self-assembled channel, a total of about 60 or 70 hydrogen bonds are formed per channel, a very sizeable enthalpic driving force.

Although other synthetic self-assembling cation channel structures have been reported^{9,10}, only a few⁹ have been evaluated using patch-clamping techniques as Ghadiri and his colleagues do here. The patch-clamp technique affords a direct measurement of the ion current through a single channel structure in a flat bilayer segment held at the tip of a capillary pipette. It is clearly the best way to

assess the relative efficiency of different cation transporting channels incorporated in lipid bilayers. In the absence of direct structural proof of the proposed channel, this technique offers convincing evidence that the structures are present and establishes their cation transporting efficacy beyond doubt.

Given the easy and selective control of the diameter of these self-assembled cylindrical β -sheets, many possible biological uses can be envisaged — regulating the flow of a variety of ionic and neutral species is essential in cell biology. In future, it may be useful to incorporate gates in these structures, to allow control of their 'open' and 'closed' positions. It seems to me that these authors are on the right track to the preparation of a series of self-assembling structures with unusual, and perhaps even selective, transport properties across lipid bilayers. We should look forward to more channel structures based on the same principle. □

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ECOLOGY

Mistletoes' close embrace

Peter D. Moore

WRITING about the European mistletoe (*Viscum album*) in 1882, the German plant physiologist Julius von Sachs¹ postulated that "its roots penetrate, as one may assume, only to absorb water and minerals dissolved in it, which is being conveyed in the wood of the tree to the leaves; whether the roots . . . extract organic matters also from the tree is uncertain".

J. D. Marshall and colleagues² are the latest group to revisit this question. They present a study of Australian mistletoes and their hosts, in which they examine the extent to which these epiphytic hemiparasites use their hosts simply as a source of water or as a supplementary source of organic carbon. Moreover, they also tackle the matter of whether mistletoes match their growth rates to those of their hosts, and whether their high transpiration rates reflect their need for nutrients such as nitrogen which are tapped from the sap of the host.

All mistletoes contain chlorophyll and conduct their own photosynthesis, but they vary in the extent to which they rely upon their host for organic materials. Some species have haustoria (Sachs's 'roots') that penetrate into the xylem vessels only, whereas others also tap the phloem and therefore have easier access to the products of host photosynthesis. Among the xylem-tapping species, the

assumption of Sachs that water (plus inorganic ions) is the main target of the haustoria has been well supported by physiological studies, including an early experiment in which coloured dyes were used to follow the course of water from host to parasite³. Many of these studies also show that the parasite is profligate in its use of the stolen water, its transpiration rate frequently being several times higher than that of the host⁴.

Experiments to follow the movement of carbon compounds from host to parasite have relied mainly on the use of the radioactive isotope ¹⁴C. Such work has shown that the phloem-tapping species gain a substantial portion of their carbon in this way, which accounts for their having lower chlorophyll densities than their hosts. Xylem-tapping species are richer in chlorophyll and seem to gain little carbon from their hosts⁵.

The photosynthesis : transpiration ratio in mistletoes is often low, mainly because of their high transpiration rate. Marshall *et al.* studied 11 species of Australian xylem-tapping mistletoes displaying this low ratio, a feature which is all the more surprising given the arid conditions under which these species exist. You might expect a plant living in dry conditions to develop a conservative water-use strategy, resulting in a high photosynthesis : transpiration ratio. A possible explana-

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tion is that the high transpiration rate enables the parasite to divert and concentrate elements such as phosphorus and nitrogen from the sap of the host, for mistletoes that parasitize nitrogen-fixing hosts transpire less⁴. The high transpiration rate may also, of course, provide a means of taking up more organic carbon from the host's xylem.

One way of checking how much carbon the parasite is obtaining from the host is to measure $\delta^{13}\text{C}$ levels in its tissues. The carbon isotope ratio within a plant is closely related to the daytime concentration of CO_2 within its tissues, and this differs between host and parasite. So one can calculate an expected $\delta^{13}\text{C}$ level based on the parasite's own photosynthesis, but this will be modified if there is uptake from the host. In fact, Marshall *et al.* show that the host contributed about 15 per cent of the total carbon gain of the parasite (averaged over all the species studied). So the answer to Sachs's uncertainty is that xylem-tapping mistletoes do take some photosynthate from their hosts.

When the parasite's total carbon gain (that is, photosynthesis plus carbon taken from the host) is compared with the photosynthetic rate of the host, the result is a simple positive correlation. This suggests that the growth of the parasite is closely adjusted to the growth of the host plant, which makes good ecological and physiological sense. If the parasite grew too fast, it might find itself unable to tap enough xylem from its host's support branch to provide it with an adequate water supply; the consequence would be wilting and death for the mistletoe. If it failed to grow fast enough, on the other hand, the mistletoe could easily become shaded by the host's leaves and might become unable to photosynthesize fast enough to keep itself out of carbon deficit. Linked into this balance is the need for the mistletoe to maintain a leaf area, and therefore transpiration rate, at a sufficient level to supply nitrogen and other essential elements for its support from the host xylem.

So a complex picture is emerging from research on this group of plant hemiparasites. Clearly, their relationships with their hosts are as delicately and finely tuned as those of equivalent organisms in the animal kingdom. □

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'Fireflies' on the Sun

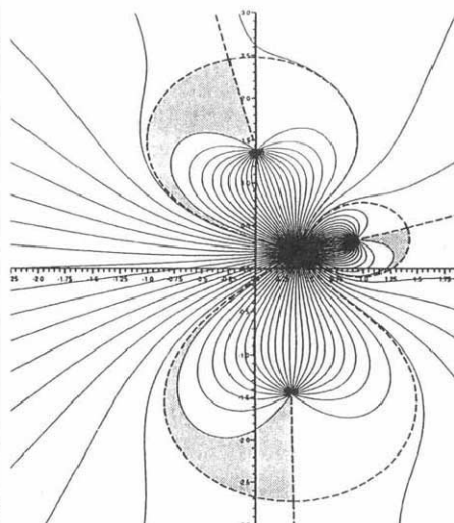
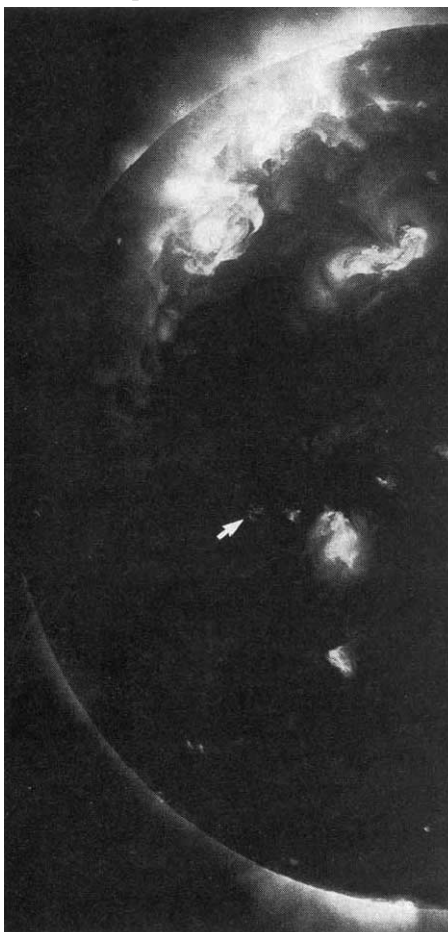
T. G. Forbes

ONE of the remarkable discoveries made by the first solar X-ray telescopes launched into space in the 1970s was the existence of dozens of bright, point-like sources dotted across the surface of the Sun. The sources wink on and off at random locations like fireflies above a meadow on a warm spring night. Ever since their discovery, researchers have speculated that the bright spots are somehow produced by the annihilation of small portions of the Sun's magnetic field; but it has never been clear why annihilation should occur intermittently at random locations. Now a group of British and American researchers has proposed a simple model which provides an elegant explanation of how magnetic field annihilation can lead to such impulsive sources. One paper appeared in last week's *Astrophysical Journal*¹; further details are to appear in *Solar Physics*².

According to the new model, the on-off

behaviour of the sources (or bright points, as they are commonly called) arises from the sudden appearance and disappearance of an 'x-point' topology in the solar magnetic field. The name refers to the x-configuration which is formed by lines of magnetic force when magnetic fields of opposite polarity meet. In 1953, J. W. Dungey of Imperial College London showed that an x-type topology would necessarily lead to the annihilation of the magnetic field and to heating in a highly ionized atmosphere like the Sun's³. On the Sun, the magnetic energy released by this process is so enormous that the solar atmosphere can reach temperatures of just over a million kelvin, which is exactly the temperature required to produce the X-ray emission from the bright points.

Previous models have assumed that x-points are continually present but that annihilation was switched on and off by some unknown process. The new model



Bright sparks. Left: X-ray image of the Sun's disk. Most of the emission is continual and comes from large regions close to sunspots. However, the arrow shows a transitory X-ray bright point which has just switched on. Top right: blow-up of this bright point showing filamentary fine structure. Bottom right: location of the lines of force extrapolated from observation of the surface magnetic field at the moment when the bright point was present. At this time there are three x-points at the locations where the dashed lines intersect. The shaded region shows the location of the heated filaments predicted by the new model.

L. Golub

C. E. Parnell & E. R. Priest

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RÉSUMÉ

requires no such process because it assumes that the x-point is only intermittently present. By combining an analytical procedure for extrapolation of the surface magnetic field with high-resolution observations of the actual magnetic field, the Anglo-American team has shown that collisions of numerous tiny magnetic regions on the solar surface sometimes produce a short-lived x-point just above the Sun's surface. In previous work⁴, some of these magnetic regions, normally referred to as 'ephemeral active regions', have been observed to approach each other and disappear. Although the disappearance of the ephemeral regions is itself fairly slow and steady, it can nevertheless cause an x-point to pop out of the solar surface, move upwards a short distance into the atmosphere, and then drop back into the surface in a matter of minutes.

Additional support for the new model comes from very-high-resolution images of X-ray bright points obtained with an advanced technology X-ray telescope during a rocket launch at White Sands, New Mexico, in July 1991 (ref. 5). One of these images is shown in the figure on the previous page, together with a blow-up of

one of the bright-point sources. The expanded image shows thin filaments of superheated gas extending outwards from a central region. These filaments are neatly explained by the new model as lying along magnetic lines of force which are directly connected to the x-points. So the new model successfully accounts for the fine structure, as well as the intermittency, of the X-ray bright points.

What is needed to confirm this new model is numerical simulation of the detailed plasma dynamics as the x-point appears and disappears. Coupled with higher-resolution images at different X-ray energies, such simulations should make it possible to prove or disprove this concept. □

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DEVELOPMENTAL BIOLOGY

The inducer that never was

J. M. W. Slack

KING Lear may have said "Nothing will come of nothing". But had his daughter Cordelia been a developmental biologist she might have inherited the anterior third of the nervous system, and her more obsequious sisters been left with the trunk and tail. This surprising outcome is suggested by work on neural induction in *Xenopus*, published last month in *Cell*^{1,2}, in which Melton and colleagues show that neuralization can arise not from the presence of an inducer but from its absence.

When I first started working on induction in early embryos, quite a few people said to me that it was a waste of time because "Everyone knows that half the substances off the shelf will do it". They were thinking of the notorious gold rush for the neural-inducing factor in amphibian embryos³. This had started in 1932 when four German laboratories simultaneously announced that killed dorsal lip tissue would induce neural tissue from gastrula ectoderm. There was a scramble to isolate the chemical factor responsible, but curiously each lab ended up with a different type of substance (protein, nucleic acid or lipid). Soon afterwards it was found that various pure substances, some of which did not occur in nature, would provoke neural induction, and interest in the problem waned because it was felt that it would not be possible to pinpoint a

specific substance as responsible for such an unspecific process.

The older work was done with newt embryos, whereas the recent renaissance of inducing-factor research has used instead the African clawed frog *Xenopus laevis*. So obsessed were we with the idea that neural induction was nonspecific that for some time nobody worked on it seriously. But in the context of research on other processes such as mesoderm induction and dorsalization, people in several labs put various substances onto isolated explants of ectoderm, and it gradually dawned on us that we were not seeing masses of nonspecific neuralization; rather, we were not seeing it at all. So in *Xenopus* the 'well known' nonspecificity of neural induction was not a problem after all. In fact the absence of candidate substances seemed more of a problem.

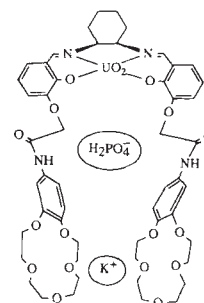
For some time, the only known treatments that would neuralize *Xenopus* ectoderm were the protein kinase C agonist TPA⁴, whose effects are somewhat marginal, and cellular disaggregation⁵, which works very well but was suspected of being some sort of artefact. Neuralization of disaggregated cells occurs at low density but is suppressed at high density, and we suggested at the time that it might be due to the dilution of an endogenous

Call of the wild

THE skulls of Arctic wolves became smaller and altered in shape between 1930 and 1950. J. Clutton-Brock *et al.* (*J. Zool., Lond.* **233**, 19–36; 1994) came to this conclusion after making a detailed series of measurements on museum specimens, and then asked "Why?". After mulling over various explanations, they conclude that the change in skull morphology was probably due to a burst of hybridization with huskies. Wolf skulls from the period 1930–50 are similar to that of a known wolf–husky hybrid; and the authors argue that, with the advent of the motor-sledge, numbers of huskies were made redundant and took to the wild — there to mate with wolves.

Pair shaped

DESIGNING a molecular receptor that will pick a particular cation species out of solution is a chemist's favourite pastime: the trick is to synthesize a molecule whose nucleophilic binding sites complement the chosen cation. The molecule here, synthesized by D. M. Rudkevich *et al.*, goes one better: it



selects both halves of the biologically useful salt KH_2PO_4 , the crown ether jaws grasping the K^+ cation whilst the central cleft binds an H_2PO_4^- anion (*Angew. Chem. int. Edn Engl.* **33**, 467–468; 1994). The hope is that receptors like this will be good for selective transport across membranes and in sensor technology.

Lend a hand

D. PURVES and colleagues have come up with another puzzle for those exercised by the matter of right- and left-handedness. Exploiting Archimedes' principle, they have measured the hand size of groups of both right- and left-handers by volumetric displacement; that is, volunteers submerged their hands in a tank of water up to a defined point, and the amount of water displaced was weighed. Purves *et al.* find that whereas there is a clear asymmetry in hand size among their right-handed subjects, in whom the right hand is larger than the left, the corresponding difference is much less marked in left-handers (*Proc. natn. Acad. Sci. U.S.A.* **91**, 5030–5032; 1994). The underlying research agenda is neurobiological: the authors intend to go on and explore whether hand asymmetry or lack of it is also evident in the related neural structures.

inhibitor by the bulk medium⁶.

The latest results are actually a spin-off from work on mesoderm induction, which in *Xenopus* occurs before neural induction, while the embryo is still at the blastula stage. It has been known for a few years that activin is a highly potent mesoderm inducer^{7,8}, and Melton's laboratory designed a dominant-negative activin receptor ($\Delta 1XAR1$) which could ablate the activity of the endogenous receptor when overexpressed in embryos⁹. This did produce embryos with reduced mesoderm, although it is now known that the reagent is not specific for activin and also inhibits the action of other members of the transforming growth factor (TGF β) family such as Vg-1 (D. Melton, S. Schulte-Merker and L. Dale, personal communications). Very surprisingly, when $\Delta 1XAR1$ was overexpressed in isolated explants of ectoderm it produced substantial neural inductions⁹.

This effect is now described at greater length in a follow-up paper¹. The neural inductions are anterior in character and arise directly, without the intervening formation of dorsal mesoderm. The authors suggest that these results mean that neural induction is caused by the removal of ubiquitous and tonic levels of TGF β -like factors. The second paper² associates the effect more closely with activin itself by the use of follistatin, a protein which has a higher specificity in that it inhibits activin but not Vg-1. The follistatin gene was cloned from *Xenopus* and shown to be expressed in the prospective neural-inducing region of the embryo. When it is overexpressed in ectoderm it produces neural inductions, which like the $\Delta 1XAR1$ -induced inductions are of anterior character and formed without mesoderm.

The authors would obviously like follistatin to be the endogenous neural inducer and for it to operate by removing a low tonic level of activin from its domain of expression. But the case is not yet closed. To prove that a particular substance does a particular job *in vivo*, three criteria must be satisfied: first, the substance must have the appropriate biological activity; second, it must be expressed at the right time and place in the embryo; and third, if it is taken away then the process should fail. Follistatin satisfies the first two criteria (it works and it is there). But we do not yet have an experiment on *Xenopus* in which follistatin itself is inhibited, which might be predicted to lead to a failure of neural plate formation. The follistatin gene has been mutated to inactivity in the mouse by targeted mutagenesis, and although these mice die soon after birth, the formation of the early nervous system appears to be normal (M. M. Matsuk and A. Bradley, personal communication). This suggests that in the mouse embryo follistatin either plays no

role in neural induction, or is at least dispensable.

Although the idea of the 'inducing factor that never was' is appealing, positive neural-inducing factors are probably also at work. In the last year, two substances have finally been discovered which have neuralizing activity in *Xenopus*. One is the secreted protein encoded by the *noggin* gene, which has both dorsalizing activity on ventral mesoderm and neuralizing activity on ectoderm^{10,11}. These two properties correspond closely to our understanding of the functions of the organizer region in *Xenopus*¹², where *noggin* is indeed normally expressed¹³. The other substance is fibroblast growth factor, normally thought of as a mesoderm-inducing factor, but also able to neuralize gastrula ectoderm cells so long as they are dissociated¹⁴.

Interestingly, the new work is consistent with an older theory of neural patterning, advanced in 1952 by P. Nieuwkoop, called the 'activation-transformation theory'¹⁵. This postulated an 'activation' step at which the whole neural plate was initially induced with an anterior specification, and a 'transformation' step at which more posterior levels were induced from its neural plate by another signal from the posterior part of the mesoderm. Both *noggin* and follistatin induce anterior-type neural markers and not posterior ones, suggesting that they correspond to 'activation' factors. This is why Cordelia would have got the brain, while the silky-tongued Regan and Goneril would have received more posterior territories. The likelihood is that a complex process such as neural induction will turn out to involve quite a variety of factors, both positive and negative, and that we have so far found the first few of them. □

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DAEDALUS

Read my tyres

THE car is taking over the world. Modern economies judge their health by their motor industries; new roads are jammed as soon as they open; every city is choked with road traffic. One solution is road pricing. At the moment this requires every car to carry an expensive electronic transponder.

Daedalus has a neater idea. He points out that a car stamps a signature, its tyre pattern, on every inch of road it traverses. He plans to read this signature.

One way would be to mould an individual black and white bar code into every tyre. It could be scanned by a reader set into the road at each pricing point. The system would come into being quite automatically, as people replaced their worn tyres with the new coded ones. But individually coded tyres would be expensive. It would be cheaper to put a magnetic filler in the tyre rubber. The tread pattern could then be read magnetically by sensors in the road. Four detailed tyre patterns, each changing slowly with wear, should be an easily recognizable fingerprint.

Even better, a magnetic tyre could be given a special magnetic code, like the one on a credit card stripe. When the car was licensed, its tyres would be coded; thereafter, magnetic readers in the road could recognize it.

There would be problems, of course. The motor trade, legendary for its sharp operators, would not welcome its role as government informer and enforcer. Unscrupulous dealers would erase the coded tyres with a magnet, or record false codes on them. To counter such trickery, Daedalus's magnetic road-readers will also write on the tyres as they pass. They will mark each tyre with a code that can be checked and updated by successive readers all down the road. Every car would then leave such a detailed trail that attempted frauds and evasions could be swiftly detected.

Daedalus goes even further. A car with magnetically coded tyres could act as a smart credit card. Its driver could pay for petrol, parking, drive-in fast food, even road pricing itself, 'on the hoof', so to speak. Those simple souls to whom cars and credit cards are both mainly forms of ostentation might be happy to combine the two. Their cars would then define their identities literally as well as spiritually. The bureaucrats of the encroaching computerized state would be well pleased. Their road readers would tell them the exact location and bank balance of every significant citizen all the time. Insignificant citizens, that is, those without cars, would be even more of an underclass than they are now.

David Jones

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Clues to ancient methane release

SIR — One of the most fascinating results of the various studies on polar ice cores is the discovery of large-scale changes in atmospheric methane over the past few ice-age cycles^{1–3}. Methane is a radiatively highly active greenhouse gas⁴, and so changes in its concentration are thought to contribute substantially to climate change⁵. The role of the chief reservoirs of methane in producing atmospheric methane variations in the past is poorly understood, as is true for the present⁶. One large reservoir that must be consi-

Clearly, an inventory of methane release as a function of climatic stage (or at least the sign of any release) on the sea floor would be of the greatest interest in deciding the question about the sign of the postulated feedback. The task is perhaps not as hopeless as it might seem at first blush. Methane within the continental slope is highly depleted in ¹³C, with ¹³C-values typically near –50‰ (–35‰ to –80‰, depending on proportions of CH₄ derived from fermentation, CO₂ reduction, or thermal fractionation¹¹). If such methane reaches the sediment surface in unusual amounts, it should considerably decrease the ¹³C of ambient dissolved inorganic carbon when being oxidized in the pore space of surface sediments. In

turn, this process would be recorded by benthic foraminifera living at or just below the sediment surface. Their sensitivity to ambient ¹³C is reflected in the fact that infaunal foraminifera have low ¹³C-values compared with epifaunal ones^{12,13}.

That benthic foraminifera are indeed sensitive to the presence of methane is suggested by the extremely negative ¹³C values in the late Quaternary section (isotope stage 5) of the deep drilling site ODP 680 B, off Peru (see figure). This site is located on the shelf in a region of upwelling and up-slope from known gas-hydrate occurrence. These values define a range well beyond the usual one of about 2‰ (ref. 14). The two species analysed, *Bolivina seminuda* and *Nonionella auris*, show

parallel excursions; however, they are not sensitive to the same degree. Thus, the possibility emerges that certain species might be indicators for high concentrations of biogenic methane in pore-waters just below the sediment surface, from sources deeper in the sediment.

Could the negative ¹³C spike be a result of conditions other than the presence of methane? Conceivably, enough organic matter could be oxidized from sulphate reduction to depress the ¹³C of the CO₂ within the pore waters by more than 10‰ (at a depth of >20 cm (ref. 15)). However, if this mechanism is to be called on to provide the unusual carbon-isotope ratios in *N. auris*, we must then assume that this species builds its shell at considerable depth within the sediment, that is, within an anoxic (and presumably H₂S-containing) environment. This seems unlikely. There is, in any case, no correlation between organic carbon content (C_{org}) and ¹³C, which would be expected in the sulphate hypothesis. Instead, *N. auris* is

highly abundant during negative ¹³C excursions of *B. seminuda*. We suggest that *N. auris* is an indicator for the presence of methane and is possibly specialized in feeding on methane-oxidizing bacteria.

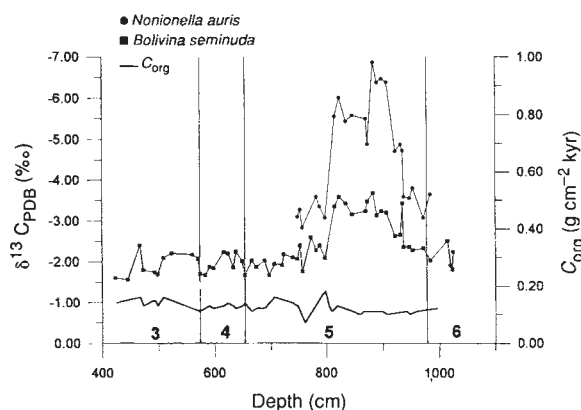
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Organic carbon content C_{org} and ¹³C values of benthic foraminifera in a core raised off Peru (ODP site 680 B, Leg 112), at a water depth of 252 m. Methane hydrates are abundant on the slope in this region¹⁶. The benthic foraminifera analysed are *B. seminuda* and *N. auris*. The ¹³C-values of both species are unusually light during oxygen-isotope stage 5 (stratigraphy after ref. 17), that is, during the last true interglacial period. The excursion reaches beyond –5‰, which suggests an influence from methane release.

dered is methane within the sea floor of coastal zones of high productivity.

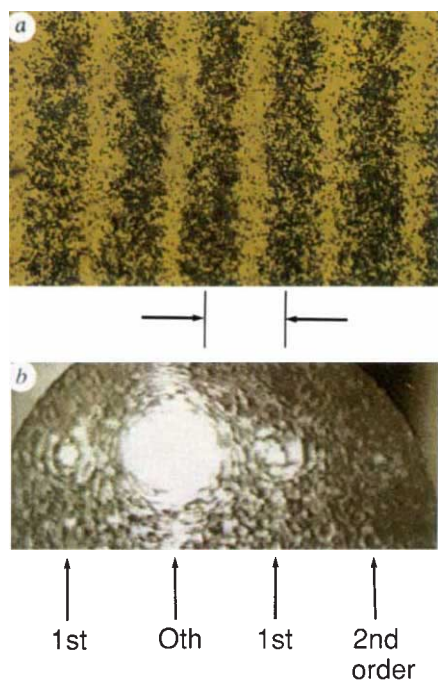
Enormous amounts of methane are thought to be stored as gas hydrates within the continental margin⁷. The stability field is such that the shallowest occurrences of gas hydrates are at water depths greater than 300 m (ref. 7). As temperature and pressure fluctuate within the sediment column (in response to ice-age climate cycles and sea-level variation), methane storage fluctuates similarly. Such changes have been invoked as a potentially strong positive feedback within the climate system, with warming leading to methane release^{8,9}. Noting that pressure change could be more important than temperature change in moving clathrates to a different position within the stability field, others have instead proposed negative feedback¹⁰. Given the ice-core results that atmospheric methane content is high in interglacials^{1–3}, a negative feedback of seafloor methane seems to imply only a minor role for marine methane in modifying ice-age climates.

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Holograms in algal suspensions

SIR — Photorefractive crystals can record holograms in the form of phase gratings arising from their response to an interference pattern. Absorption of light produces a local electric field which modifies the refractive index via the Pockels effect¹. Algae exhibiting photo-induced taxis (movement in response to a light stimulus²) can be thought of as behaving in an analogous manner, except that by their presence or absence they change the local transmission of a sample, instead of its refractive index. We report here the recording of an optical grating in a suspension of algae and the subsequent reconstruction of a simple hologram.

The flagellate *Dunaliella primolecta* Butcher (CCAP 11/34) is usually contained in cuvettes of path length 1 cm at number densities of about 10⁵–10⁶ ml^{–1}, corresponding to cultures of 2–6 weeks' growth from inoculation. Both positive and negative taxis can be seen, following illumination for periods of 15 min up to a few hours by the beam from a randomly polarized 2-mW 632.8-nm He–Ne laser. Inspection of the cuvette shows that in



a, Interference fringes of 360- μm period recorded in *D. primolecta*. Distance between arrows, 360 μm . **b**, Far field pattern showing first and second order diffraction from an algal grating.

positive taxis the algae congregate to form a spot on the input window and a less dense one at the exit, the spots being of approximately the same size as the incident light beam. In the case of negative taxis, an annular pattern is generated with a distinct inner edge, indicating that the maximum intensity for positive taxis is located at a certain radius of the gaussian profile of the laser beam.

During illumination, the transmission of the cuvette decreases to an equilibrium value indicating the existence of an intensity threshold for positive taxis of about 2.7 mW cm⁻² at 632.8 nm. In this respect, *D. primolecta* behave differently from a photographic emulsion that requires only a minimum exposure, not a minimum light intensity; otherwise, a characteristic curve of optical density versus the logarithm of the exposure may be deduced as for a film, yielding an ASA rating (film speed) of about 10⁹!

The ability of tactic cells to record holographic images by this 'printing' process is demonstrated by exposing them to interference fringes produced by superimposing two coherent light beams in the cuvette. Algal 'holograms' of high contrast as in the figure (a) can be printed onto the inner surface of the cuvette window for beams with crossing angles of about 0.9 – 0.2°. The resulting fringe periods of 75–400 μm are about 10–50 $\times$ the *D. primolecta* cell size. The increase in density of the fringes towards the lower edge of a in the figure is caused by the centres of the beams' gaussian cross-sections being below the field of view.

Gratings of this form are equivalent to the hologram of one of the original recording beams, the other acting as the reference. Reconstruction is demonstrated in b in the figure, where the second-order spots are indicative of a thin nonsinusoidal grating.

Apart from the novelty of this biological analogue of an optical process, we expect to obtain basic biophysical results. By changing the spatial period of the fringes, for example, the scale of the light-intensity gradient causing taxis can be controlled to less than a cell size. In this way, the mechanism by which cells, moving at speeds of a few millimetres per second, can distinguish between the nodes and antinodes may be examined in detail. Also, *D. primolecta* is the only species of several we assessed that exhibits a strong effect in red (632-nm) light. Phototaxis⁵, the response to non-uniform light intensi-

ty, usually requires blue-green light. In aerotaxis³, the response to the oxygen generated by non-uniform photosynthesis, diffusion is likely to prevent the establishment of a modulated oxygen distribution on such a microscopic scale. Further work is thus necessary to confirm the identity of the origin of the mechanism involved by using other wavelengths or by quenching the by-products of photosynthesis.

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Site of catabolite inactivation

SIR — Fructose-1,6-bisphosphatase (FBPase), a key enzyme in gluconeogenesis, is subject to catabolite inactivation in the yeast *Saccharomyces cerevisiae*. Addition of glucose to cells grown on a non-fermentable carbon source causes rapid inactivation of this enzyme due to phosphorylation and subsequent proteolytic degradation^{1,2}. We have previously shown that catabolite degradation of FBPase does not depend on the presence of the major endopeptidases of the vacuole, proteinase yscA and yscB³, indicating non-vacuolar catabolite degradation of the enzyme.

Chiang and Schekman described in 1991 (ref. 4) the regulated, selective transfer of FBPase from the cytosol into the vacuole on glucose addition to yeast cells depressed in FBPase, followed by degradation of the enzyme, which depends on the *PEP4* (*PRA1*) gene product, proteinase yscA. Proteinase yscA occupies a central position in the vacuole. The enzyme is necessary for activation of its own and other inactive vacuolar peptidase precursors and it is highly active in vacuolar protein degradation³. Chiang and Schekman concluded that glucose-triggered FBPase degradation is localized to the vacuole.

We repeated our previous experiments³ with a set of isogenic yeast strains. Wild-type, and for comparison, *pral* (*pep4*)-deleted mutant strains were

radiolabelled with ³⁵S-methionine, derepressed for FBPase on ethanol-containing medium and then transferred onto glucose medium to induce FBPase inactivation. Samples were taken at different time-points, cells were lysed and radiolabelled FBPase was immunoprecipitated using specific antibodies, separated on SDS-PAGE and visualized by autoradiography (see Fig. 1). As a control protein not affected by catabolite inactivation but at the same time monitoring the *pral* mutant phenotype showing a defect in vacuolar peptidase processing, carboxypeptidase yscY (CPY) was co-precipitated. Even

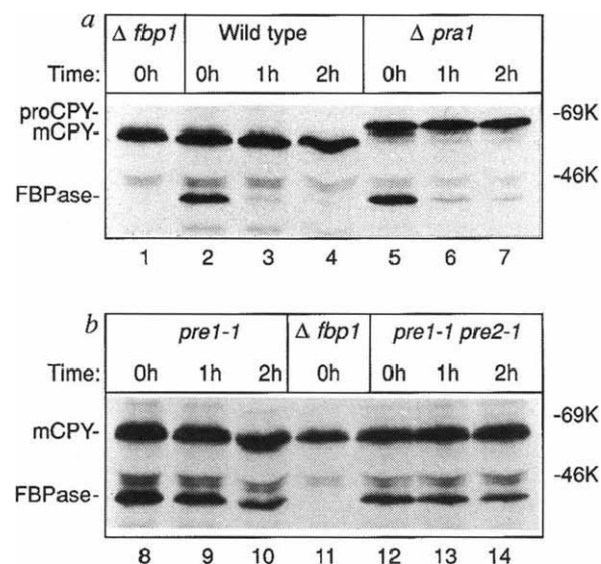


FIG. 1 Glucose-triggered degradation of FBPase is not vacuolar but depends on an intact proteasome. Lanes 1, 11, FBPase null mutant (*fbp1Δ::LEU2*) shifted to glucose for 0 h (control). Lanes 2–4, wild-type cells shifted to glucose for 0, 1 and 2 h respectively. Lanes 5–7, *pral* cells shifted to glucose for 0, 1 and 2 h. Lanes 8–10, *pre1-1* cells shifted to glucose for 0, 1 and 2 h. Lanes 12–14, *pre1-1 pre2-1* cells shifted to glucose for 0, 1 and 2 h. Numbers on the right show M_r markers.

after only 1 h on glucose, FBPase protein is almost completely lost in wild-type and the *pral*-deleted strain (part *a* in Fig. 1). From this experiment we conclude that catabolite degradation of FBPase is not dependent on vacuolar proteolysis and thus not localized to the vacuole.

The proteasome is a major, non-vacuolar proteinase, highly conserved in all eukaryotes⁵. This enzyme complex is localized in the cytoplasm and in the nucleus of cells⁵, and is involved in the degradation of ubiquitinated and short-lived proteins *in vivo*⁶. When using the same derepression and inactivation protocol for FBPase as described in part *a* of Fig. 1, we found that mutants (*prel-1* and *prel-1 pre2-1*) (part *b*) defective in the chymotrypsin-like activity of the proteasome show a dramatic retardation of glucose-triggered FBPase degradation. In the double mutant (*prel-1 pre2-1*) defective in two subunits of the proteasome necessary for the chymotrypsin-like activity, degradation of FBPase is nearly absent (part *b*). From these experiments we conclude that glucose-triggered degradation of FBPase is mediated by the proteasome.

Why do our results differ from those in ref. 4? We repeated the catabolite inactivation experiments with the wild-type and *pep4*-deleted strains (W3031B and W3031BF) used in ref. 4 under the same growth conditions. None of the experiments provided evidence for a proteinase yscA-dependent catabolite degradation of FBPase. However, when overexpressing FBPase on the multicopy plasmid, using immunofluorescence we found FBPase in the vacuole of *pral* (*pep4*)-deleted cells without glucose addition. Also after starvation for nitrogen, FBPase is found in the vacuole⁷. From this, we conclude that FBPase enters the vacuole under stress and starvation. The most plausible mechanism for this phenomenon at present is uptake of the enzyme into the vacuole via autophagocytosis⁷.

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CHIANG AND SCHEKMAN REPLY — Because of the apparent contradiction between our results and those of Schork *et al.*, we performed the basic catabolite inactivation experiment using cells (chromosomal copy of FBPase gene only) grown in six different poor-carbon-source media followed by transfer to glucose medium (see Fig. 2). Cell samples were processed and FBPase detected by immunoblotting as before⁴. Condition A is the regimen we reported earlier⁴; the other regimens have since been found to

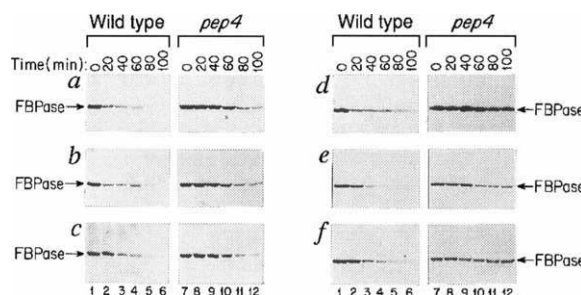


FIG. 2 Glucose-induced degradation of FBPase is dependent on the *PEP4* gene. Untransformed wild type cells (W3031B, MATa, *leu2-3,-112*, *ura3*, *ade2*, *his3*, *trp1*), and *pep4* (W303BF, MATa, *leu2-3,-112*, *ura3*, *ade2*, *his3*, *PEP4:TRP1*), were grown under various growth conditions and transferred to YP (1% Bacto-yeast extract, 2% Bacto-peptone) containing 2% glucose for 0, 20, 40, 60, 80 and 100 min. Cell extracts were obtained and FBPase examined on SDS-PAGE by immunoblotting with affinity-purified anti-FBPase antibodies⁴. *a*, YP containing 1% potassium acetate and 1% glucose for 48 h; *b*, 1% potassium acetate and 0.5% glucose for 48 h; *c*, 1% potassium acetate and 0.1% glucose for 48 h; *d*, YPD (dextrose) for 24 h and adapted to YPO (2% oleic acid) for additional 24 h; *e*, YP containing 2% pyruvate for 48 h; *f*, YP containing 2% galactose for 48 h.

be equally or even more effective in revealing the stabilization of FBPase in *pep4* mutant cells. Furthermore, we find by immunofluorescence microscopy that the alternative conditions of carbon source transfer confirm our conclusion concerning the transport of FBPase from the cytosol to the vacuole during catabolite inactivation in *pep4* mutant cells (H.-L.C., in preparation).

Schork *et al.* conclude that FBPase is degraded in the cytosol because mutants defective in two subunits of the cytosolic proteasome fail to engage in catabolite-mediated degradation of FBPase. We agree that *prel* and *pre2* mutations affect FBPase degradation, but we feel it is premature to base the conclusion of cytosolic degradation of FBPase on the phenotype of two mutants known to be pleiotropic.

Ubiquitination of protein substrates which regulates proteasome-mediated protein degradation, is required in the biogenesis and function of certain cytoplasmic organelles. Ubiquitin conjugating enzymes have been localized to the endoplasmic reticulum, Golgi and peroxisome, and protein-ubiquitin conjugates are found in the yeast vacuole^{8,9}. Note that

a mammalian cell-cycle mutant defective in ubiquitin conjugation displays deficient autophagic degradation of protein substrates in the lysosome¹⁰. We have found that ubiquitin conjugation mutants (*ubc1*, 4, and 5), as well as a variety of other mutants that affect membrane biogenesis (secretion, *sec2*, 18, 7; endocytosis, *end1*, 3; peroxisome assembly, *pas1*, 2, 3) are defective in catabolite-mediated FBPase degradation (ref. 4; our manuscript in preparation). These results are inconsistent with a cytosolic location of the FBPase degradation event, but instead suggest a ubiquitin-regulated autophagic process whereby FBPase is sequestered in an autophagosome which is

then degraded in the vacuole. This model accounts for our observation that FBPase is transported from the cytosol into the vacuole when *pep4* mutant cells are transferred into glucose medium⁴.

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Invastion test

SIR — Butcher and Deng¹ state that I argued in my News and Views article² that high inbreeding would be required to prevent the invasion of SisterKillers. They go on to show that this assertion is wrong. I however made no statement of the required invasion conditions. I did argue that to test Haig's model³ it would be useful to examine heavily inbred organisms as these are the least vulnerable to SisterKillers. I appreciate that Butcher and Deng analytically show that this statement is correct. Although Haig's proposal would be strengthened by a dynamic analysis, his argument is well founded, and Butcher and Deng are mistaken to say that my acceptance of its logic was premature.

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Super natural selections

Peter Tallack

Darwin and Darwinism: An Exhibition on Cultural and Natural History. Deutsches Hygiene-Museum, Dresden (tel: +49-351-4846-209). Until 26 June 1994.

"LET us hope that it is not true; and if it is true, let us hope that it does not become widely known", declared Lady Ashley, Darwin's contemporary, on learning of his theory of mankind's descent from ape-like creatures. But it was and it did — one wonders what this wonderfully provincial lady would have made of this superb exhibition, a celebration of the diversity and influence of evolutionary ideas.

The scope, both across time and across disciplines, is vast. Some 600 items, spanning half a millennium, have been gathered from around the world and organized to show how our perception of nature has itself evolved over the ages.

Many are unique, such as the late-eighteenth-century 'Holzbibliothek' or 'wood library' of Carl Schilbach, each volume fashioned almost entirely from a particular species of tree or shrub, and the selection from Frederik Ruysch's 2,000 anatomical specimens, which Peter the Great bought for 30,000 Dutch guilders and transported *en masse* to Russia. Among the human oddities are a deformed infant's foot ornamented with an allegorical scorpion, and an abnormal fetal body, head and arm decorated with lace — this is the first time that these evocative relics have left St Petersburg since their arrival there in 1717 (they were once thought to have been lost after Georges Cuvier spread the rumour that sailors had drunk the alcohol in the preserving jars).

An enormous variety of prints, portraits, drawings and paintings introduces the players in the events leading up to, and set in motion by, Darwin's intellectual bombshell. Here are Linnaeus's *Systema Naturae*; Eldin's pen-and-ink drawings for Hutton's *Theory of the Earth*; Thomas de la Beche's whimsical cartoons of the prehistoric world; Benjamin Waterhouse Hawkins's plans for his ill-fated New York dinosaur park; Wallace's notebooks and sketches from his Amazonian expedition (virtually everything else from this four-year voyage of discovery was lost at sea); Owen's diagrams of archetypes and homologies of vertebrate skeletons; Haeckel's sepia drawing of the evolutionary tree of life; Galton's composite photography; and Weismann's lovely unpublished watercolour studies of the origin of sexual cells in *Hydroida*.

The cast also includes charlatans (a section charts the corruption of genetics and evolutionary theory under Stalin), fakes such as the 'freaks' and 'missing links' of P. T. Barnum's American

Museum, shown in five daguerreotypes, and the famous Piltdown Man skull hoax of 1912. Some lesser-known palaeontological skulduggery occurred two centuries earlier when Johann Beringer, a German geologist, fell victim to 'Lügensteine' or 'fossil lies'. The more improbable his find,

tual highways and byways. The cultural, social and political backdrop is expressed in the form of paintings and photographs of key places and events, several period costumes and even a model steam engine.

Evolution as a concept has taken a great deal of abuse down the years. Take the nineteenth-century Italian criminologist Cesare Lombroso, the subject of one display, who claimed that 'criminals' (his definition embraced prostitutes and revolutionaries) are trapped in early stages of human development, or are throwbacks to a primitive type. To try and prove his point he made a large collection of crimi-

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'Squid' and 'Gorilla' — costume sketches for a New Orleans Mardi Gras parade on the theme of 'Missing Links'. The series satirized a hundred well-known people, mainly generals, politicians and government officials. President Abraham Lincoln appears as an ass, whereas Harriet Beecher Stowe, author of *Uncle Tom's Cabin*, is portrayed as a leopard. Charles Briton, 1873.

the more excited he became. He dutifully described the 'fossils' in *Lithographiae Wirceburgensis*, published in 1726. Shortly after, he found a stone that gave the game away — it supposedly had his name on it — and he spent the rest of his life buying up and destroying copies of the book, making the one exhibited next to the stone collection a great rarity.

It is of course Charles Darwin who presides. Various items hint at the transformation of the energetic young naturalist who sailed aboard the *Beagle* into the reclusive semi-invalid philosopher. On show are nautical instruments, maps and drawings from his earlier life, together with knick-knacks, many from Down House, his home for 40 years, such as a copy of *Das Kapital* given to him by Marx (and inscribed from a "sincere admirer") and the walking stick that supported him on his daily strolls along the 'Sandwalk', his 'thinking path'.

But this exhibition is more than a guide to the historical personalities and concep-

nals' death masks, prison graffiti and tattooed skin (visitors will encounter a tattooed penis among these bizarre samples). For decades his theories were taken seriously, by serious people, and his unwholesome influence still lingers.

Galton casts an even longer shadow. Devices for measuring skull angles, as well as US Army medical tests and cards for assessing the literacy of immigrants, accompany several posters tracing a variety of eugenics programmes, including the attempts to create a 'master race' in Nazi Germany. The bleakness of this section is chillingly poignant: the Deutsches Hygiene-Museum, host of the exhibition, was established in 1911 to improve public health and industrial hygiene in the spirit of the eugenic approach popular at the time; and under the regime of National Socialism, it served as a centre for Nazi propaganda. Especially striking are the life-size statues of 'ideal' racial types (including a glorified 'Nordic Man'), Austrian replicas of those commis-

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

First naturalistic picture of a monkey, 15th c.

sioned by the Museum of Ethnology in Dresden for the German Colonial Exhibition of 1939, and destroyed in the bombing of the city in 1945.

All of this makes for a visual treat. In many of the exhibits science shades into art, as in the delicate sketches of fish made by the artist Jacques Burkhardt during Agassiz's expedition to Brazil (one of his other assistants was, incidentally, William James) and Gould's and Audubon's bird paintings (Audubon, whose name is today adopted by a leading US conservation organization, in fact shot up to 20 specimens for each plate in his *Birds of America*). Also stunning are Gessner's and Ehret's botanical illustrations, which for centuries have remained hidden, largely uncatalogued, on the shelves of museums and herbaria. Yet the best of the art merely imitates life — examples of the real thing here include Haeckel's corals, 46 human skulls from Virchow's anthropological collection, the skeleton of a 21-foot python, an elephant's skull studied by Goethe, and a stuffed menagerie of feather and fur.

The exhibition has gaps, both in disciplines (geology, palaeoanthropology and embryology are surely underrepresented) and in personalities (Lyell, Dart and Haldane arguably deserve greater recognition, for example). These deficiencies are in part redeemed by the informative German-language catalogue, and all in all *Darwin and Darwinism* is a memorably successful survey of the genesis, sweep and power of a scientific idea. The pity is that the chance to see it has almost gone. The show will not go on the road, and from the end of June the exhibits will begin to return to their homes. □

Peter Tallack is Book Review Editor of *Nature*.

Pilgrimage towards truth

Dai Rees

To Be a Scientist. By Donald Braben. Oxford University Press: 1994. Pp. 166. £9.99 (pbk).

SCIENCE is a vocation to which we are called, a way of life and system of values, and a pilgrimage towards truth about aspects of our world. It calls for a level of honesty, rigour and sheer hard work that many find difficult to sustain. It organizes itself into convocations, societies and establishment groups that sometimes confuse their own greater glory with that of the noble cause. Can it then be surprising if the practice of the scientific life leads to a diversity of beliefs and doctrines similar to those evident in the history of religions down the ages? We study and revere the lives of our saints in science, draw lessons from the downfall of sinners and ask ourselves why it is that the wicked so often prosper. One illustration of the link between the emotions of science and religion is in the story of the great Ernest Rutherford who, when excited by a particularly good research result, would lead his team in a march around the laboratory benches to the refrain of "Onward Christian Soldiers".

Whether intentionally or not, Donald Braben has given the title of his book a Bunyanesque ring that will appeal to many a romantic soul, as it does to my own. I can agree with him that there is much wickedness in those parts of the scientific establishment that come under his attack. Perhaps his favourite example is from aspects of the awards system for so-called 'responsive-mode' research grants, but unfortunately this system is itself bolstered by a substantial body of fervent quasi-religious support. To dismantle such heresies needs the application of intellectual incisiveness (which I don't find in the book), rather than assault with slogans that appeal only to the converted. "No Popery here" can have a stirring resonance, but only if you happen to be on the same side. Nor do I find convincing the doctrine that he then goes on to develop as a substitute, prescribing liberation of the movers and shakers and more trust in the inspiration and unpredictability of the scientific equivalent of the Holy Ghost. It is probably too early for definitive evaluation of Braben's own BP Venture Research Programme but he does seem to have had a significant degree of success. Examples of its activities are recounted in a breezy entertaining style, but again the appeal to the reader is not to scientific reason but to the romance and supposedly self-evident virtue of doing something for the sake of being different. It is difficult to see how in practice the scheme might be generalized (no specific proposal is

made). In the chapter for aspiring young scientists, life in the system is painted as harsher and in a more unsympathetic world than I have found it to be; is there just a hint of disillusion here?

Despite these quibbles, Braben is right to identify as the crucial issue of our time the question of how we identify, support and develop scientific talent. Preaching about the need for a scientific renaissance is unlikely to be any more successful than was merely praying for revival of religious passions of the past, as in the Nonconformist Wales of my childhood. The message has to match the needs of the time. Part of the right message has, I think, been captured in general outline in the UK government's recent White Paper (policy document) *Realising our Potential*. We need to demythologize science to reveal its meaning in terms of the everyday life of the public, the economic aspirations of political masters, the curiosity and career aspirations of the adventurous young; and we need to deliver and be seen to deliver effective and stimulating training, rewarding careers and the promotion of research that will lead to exciting insights and

New in paperback

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Pimlico, £12.50. "Read this book and you will never see the world around you in quite the same way again" (Laura Garwin, *Nature* **368**, 366; 1994).

Perilous Knowledge: The Human Genome Project and Its Implications by Tom Wilkie. Faber, £7.99, \$12.95.

"Wilkie writes accurately, forcefully and unsensationally. For those who want an accurate, but not too technical account of what is happening in genetics, and the ethical problems to which the discipline gives rise, *Perilous Knowledge* could scarcely be bettered" (John Habgood, Archbishop of York, *Nature* **365**, 304; 1993).

Pietà by George Klein. MIT Press, \$14.95, £13.50. A biologist's collection of memoirs and reflections that "once started, is impossible to put down... no science library that aspires to more than the narrowest interpretation of its function can afford not to purchase this volume to place alongside its Jacob, its Müller-Hill and its Medawars" (N. A. Mitchison, *Nature* **360**, 388; 1994).

The Computational Brain by Patricia S. Churchland and Terrence J. Sejnowski. MIT Press, \$19.95, £17.95.

useful knowledge. There is little doubt that the survival, let alone the renaissance, of science, nationally and internationally, will require our ability to evolve successfully in these directions.

Unfortunately, such objectives are much easier to define and agree upon than are the means of achieving them, and in the implementation we see much reenactment of the building of the Tower of Babel. Witness the notorious and all too visible conflicts over the planning, introduction and management of the Framework programmes of the European Union. Nor are these problems peculiar to the Brussels administration alone — they are mirrored to a greater or lesser extent within the science politics of most if not all individual countries. The debate becomes further confused when divergent viewpoints arise, as today they inevitably do, not only from within the scientific community but also from politicians, civil servants, businessmen and others, who all feel entitled, no doubt rightly, to press the value of their own particular enlightenments and certainties.

Like so many others, I too have prescriptions. Research missions need first to be defined. Then they need to be implemented through an effective and creative management of strategy and funding that is firmly based on detailed knowledge and experience of the dynamics within research communities, and on understanding and sound judgement of the talents and motivations of people. The current preoccupation with proposing organizational structures from models of markets or theories of corporations can, I agree, be most valuable in clarifying different functions in the organization and the relationships that should exist between them. Merely to define the relationships is, however, no substitute for establishing excellence in their management and is a trivial task in comparison. Hand-waving about structures and models is currently contributing more confusion than progress. It might sound self-evident but does not appear to be so, that when faced with a choice between a structure that is fashionable or politically correct and one that would better deliver the vision of scientists, then the latter must prevail. We are threatened by a widespread and dangerous illusion that structure-definition provides a short cut to avoid hard work and the need for professional judgements of the highest quality.

The age of ecumenism is long overdue among the religions of science. Before we can formulate an honest consensus, however, we do need to sort out the gold from the dross in the different persuasions. May the quest continue. □

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JENS Munk's harbour in Hudson Bay, where 61 of his 64 companions died from scurvy in 1620 in their attempt at the North-West Passage. The picture, which appeared in his journal published four years later, is from *The Arctic: A History* by R. Vaughan. Alan Sutton, £20.

Meaning and rationalization

Ian Stewart

The Sexual Metaphor. By Helen Haste. Harvard University Press/Harvester Wheatsheaf: 1993/94. Pp. 302. \$24.95, £50 (hbk); £17.95 (pbk).

HUMANS are not so much rational as rationalizing animals. Our brains have evolved to make quick-and-dirty judgements about the likely pattern of events unfolding around us, because survival depends on our ability to outcompute our competitors. This evolutionary arms race has given us astonishing powers of pattern recognition — just the first few layers of cells in the visual cortex are superior to the fastest computers and the cleverest software. It has also given us a tendency to impose patterns where none exist, such as the identification of clusters of stars — seen in projection and not, in actuality, physically associated — with mythical animals and people.

Poised ambiguously between these two extremes is one of our most powerful pattern-classifying tricks: metaphor. A metaphor is a high-level similarity between different things or different processes. It can reflect a deep structural resonance or merely a superficial resemblance. Many of our most basic cultural assumptions rest on the foundations of metaphor, which is why slogans are such a potent political weapon. Metaphors are incisive and misleading, valuable and dangerous.

We are sexual animals, and the

metaphors of sexuality are especially deep-rooted. To quote Helen Haste's prologue: "It is extremely convenient to make assumptions on the basis of whether a person is male or female. . . . By mapping masculine and feminine on to so many other dimensions to which we also ascribe polarity, we deepen the problem. When I give lectures on this, I put up a list of these polarities and ask people to classify each [as masculine or feminine]. Everyone can do it — even though their rational selves resist stereotyping. Examples are *light-dark*, *public-private*, *arts-science*, *rational-intuitive (and rationality-chaos)*, *sun-moon*, *active-passive*, *hard-soft*, *thinking-feeling*. I invite you to try the experiment." Stop reading and do so. Try it on your colleagues and compare the results. Randomize the ordering within each category, it makes no difference. A hundred per cent agreement is the rule.

The Sexual Metaphor is a thoughtful and original examination of the effect that the common but tacit perception of these sexual slogans has had, and more crucially is having, on a society that is struggling to separate issues of sex and gender. In particular it concentrates on public-private, active-passive and rationality-chaos. Our cultural slogans are the product of centuries of social evolution, and lie at the 'izing' end of the rationalizing axis. They come from the dark, irrational depths of the human mind, and are resistant to rational thought. They can subtly, or crudely, redirect the thinking patterns

of even the most fiercely and proudly rational of us, without anybody noticing, least of all ourselves. (For example, how many of you are now holding a stereotype — 'feminist' — at the forefront of your minds, and using it to decide whether to continue reading this review? Come on, be honest.)

Traditional feminism accepts a masculine metaphor of rationality, and fights against the exclusion of women from the resulting worldview. Haste prefers to challenge the worldview itself. A special case that should be of particular concern to all scientists is to what extent the methods and objectives of science are biased by unspoken and unperceived cultural slogans about the male-female polarity.

Scientists typically take the position that science is a rational activity and that it is free of cultural biases. They hold that there is no such thing as masculine science or feminine science, any more than there is Western science and Eastern science. There is only Science — the formulation of rational hypotheses and their confrontation with experiment. In some sense this is surely true. For example, if scientific discoveries derived from an Eastern cultural background were in serious conflict with scientific discoveries derived from a Western cultural background, they couldn't both be right, and the rational scientific method should be capable of distinguishing between them. But this is the least interesting sense, because it describes an abstraction. Real scientists are people, and their cultural backgrounds bias them in the one place they seldom probe: not their experimental or theoretical methodology, but their choice of what problem to apply the methods to.

For example, in recent years there has been an enormous growth of environmental science. It is indeed the same science that went before, in the sense that the chemistry of chlorofluorocarbons is the same whether the problem is to devise a propellant for aerosols or to save the ozone layer. But think how different the consequences would have been if the science of the ozone layer had been attacked first, instead of that of aerosols. Think how different the development of that area of science would have been. Then ask yourself whether scientists can possibly pursue all avenues simultaneously; and since they can't, decide what you mean by 'science' as an abstract, acultural, ahistorical activity. Finally, ask yourself what relevance any 'sameness' in your answer can have to human concerns, especially your own.

Science in the abstract is a rational process, but one that has been tacked on to an intuitive one in order to limit human folly. It does not totally eliminate it, because humans never fully attain their ideals. But science wouldn't work if it

were as purely rational as the conventional model holds. Every area of science has its accepted paradigms, which channel the directions that investigations pursue. It cannot advance without paradigms — it would be lost in an overwhelming morass of data. But paradigms do not have a wholly positive effect. Until very recently the study of animal behaviour was dominated by the behaviourist paradigm, which denied animals any vestige of wants, intentions, emotions or consciousness. Animals were organic machines operated by drives. This is a curious paradigm to come from minds that actually 'live inside' an animal and which would be appalled to have the same description applied to themselves; and it has seriously limited our understanding of animals.

The Sexual Metaphor is a thought-provoking and brilliantly targeted book. I particularly recommend chapter 11, "Science and Rationality", to anybody who wants to open this particular mental Pandora's box. I would recommend it even more to anybody who would prefer to have the box kept shut — but I know they wouldn't take any notice. □

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Pillars of cosmology

David N. Schramm

Cosmic Questions. By Richard Morris. Wiley: 1993. Pp. 200. \$24.95.

COSMOLOGY truly is in a Golden Age. Never before have so many different kinds of experiments and observations been able to lead to conclusions about the entire Universe. Experiments on satellites, on balloons at the South Pole and other remote places are all mapping fluctuations in the cosmic microwave background radiation left over from the Big Bang. Telescopes, such as the new 10-m Keck telescope in Hawaii, are finding light elements in very primitive material as predicted by Big Bang cosmology, as well as mapping out the distribution of galaxies in the sky and finding huge structures, such as great walls, great attractors and superclusters. Researchers are building detectors deep underground to look for exotic dark matter passing through Earth. And satellites are searching in X-ray and gamma-ray regions of the spectra, probing the emission of galaxies in these non-optical wavelengths.

Just as physicists at the turn of this century acquired technical capabilities to study the structure of the atom, so now has cosmology progressed from a philosophical, theoretically based field to a true

branch of physical science grounded in experiment and observation while still maintaining a close interplay with theory. Richard Morris accurately describes the nature of this revolution, which, he points out, has not overturned the standard cosmological theory of the Big Bang, but rather has added to its richness and led to a new round of questions at a much deeper level. He calls these and others the "cosmic questions".

In the past decade, there have been many popular books on cosmology. Some have emphasized the personalities as much as the science, such as Denis Overbye's well-written *Lonely Hearts of the Cosmos*. Some have been autobiographical, such as George Smoot's *Ripples in the Cosmos* (co-authored by Keay Davidson). Others have focused mostly on the description of the science itself.

Morris's book falls into the descriptive category, and it has the advantage of being very recent and therefore up to date in this rapidly changing field, covering, for example, the recent measurements of anisotropy in the microwave background radiation. Unfortunately, Morris, not being an active player in the field, occasionally gets details garbled. He mistakenly talks about a 'critical density universe' being one that expands to a fixed size. He misses the main problems of the popular cold-dark-matter model of structure formation, yet rightly points out that many cosmologists have questions about the model. I was pleased to note that Morris recognizes nucleosynthesis as one of the three pillars of the Big Bang along with the Hubble expansion and the microwave background radiation. But for some reason, he does not specifically mention the key figures in the nuclear area, although he bends over backwards naming very minor players in the more astronomical topics.

The book ends with the cosmic questions: What is the Universe made of? How did it begin? How will it end? What is time? Some of these long-standing puzzles are finally being attacked with real experiments and data. The author also delves into more philosophical questions, discussing, for example, the anthropic principle while asking "Why is our universe so hospitable to life?"

A useful feature of this book, and one unfortunately still rare in works of this type, is the inclusion of a comprehensive glossary. This should make the book much more accessible to the lay reader or the intelligent high-school student who wants to learn more about the excitement of modern cosmology. □

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Inactivation properties of voltage-gated K⁺ channels altered by presence of β -subunit

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Structural and functional diversity of voltage-gated K_v1-type potassium channels in rat brain is enhanced by the association of two different types of subunits, the membrane-bound, pore-forming α -subunits and a peripheral β -subunit. We have cloned a β -subunit (K_v β 1) that is specifically expressed in the rat nervous system. Association of K_v β 1 with α -subunits confers rapid A-type inactivation on non-inactivating K_v1 channels (delayed rectifiers) in expression systems *in vitro*. This effect is mediated by an inactivating ball domain in the K_v β 1 amino terminus.

POTASSIUM channels fulfil important functions in many signal transduction pathways in the nervous system. They are major determinants of membrane excitability, influencing the resting potential of membranes, wave forms and frequencies of action potentials, and thresholds of excitation¹. Two major classes of voltage-gated potassium channels (K_v channels) have been described. To the first one belong the slowly inactivating, delayed rectifier-type K_v channels which have a stereotypic time course of activation and inactivation. They are involved in action potential repolarization and attenuation of cell excitability. The second class comprises the rapidly inactivating (A-type) K_v channels, which operate in the subthreshold range of action potentials and play a key role in encoding pre- and postsynaptic nervous signals². The expression of diverse A-type channels may help different types of neurons in the specific temporal integration of synaptic inputs and in the firing with distinct spike frequencies^{1,2}. Moreover, regulation of A-type channel activity may be involved in neural mechanisms related to behaviour and learning³. The importance of A-type channels suggests that they represent a major type of K_v channel in the nervous system¹. Surprisingly, however, most K_v channels cloned and characterized *in vitro* express non-inactivating, delayed rectifier-type channels⁴⁻⁶. We report here that delayed rectifiers can be converted to A-type K_v channels upon association of the pore-forming α -subunit with a peripheral β -subunit. Coexpression of a β -subunit (K_v β 1) with members of the *Shaker*-related rat K_v1 (RCK) channel family⁷ in the *Xenopus* oocyte system causes a conversion to a rapid inactivation mode of the resultant hetero-oligomeric K_v channel. This conversion is determined by an inactivating domain present in the K_v β 1 amino terminus. These studies suggest that association of α and β K_v channel subunits can modify channel-gating properties to produce plastic changes in neuronal signalling.

β -subunit of voltage-gated K⁺ channel cloned

A current hypothesis proposes that mammalian K_v channels are tetramers of similar or identical α -subunits belonging to a superfamily of ion-channel-forming polypeptides⁸. It has been suggested that homo-⁷ and hetero-multimeric^{9,10} assembly of α -subunits may contribute to the enormous K_v channel diversity found in the mammalian nervous system, but this oversimplifies the structure and arrangement of the subunits forming K_v channels. Purified rat¹¹ as well as bovine¹² α -dendrotoxin (DTX) receptors consist of two different types of subunits: membrane-

rat K _v β 1	MQVSIACTEHNLSKRNEDRLLSKQSSAPNVNNAARAKFTVIAIARSLGTFTPQHHS	60
rat K _v β 2	MYPESTTGSFARLSLRQ-GS-GMIY-	26
bov K _v β 2	MYPESTTGSFARLSLRQ-GS-GMIY-	26
rat K _v β 1	LKESTAKQTGMKYNLNGSKGLRVSCGLGLTGWTFGGQISDEVAERLMTIAYESGVNLFDT	120
rat K _v β 2	TRYGSP-RQLQF-----T-M-H-L-DN-I-----	86
bov K _v β 2	TRYGSP-RQLQF-----T-M-H-L-DN-I-----	86
rat K _v β 1	AEVYAAGKAEVILGSIKKKGWRRSSLVITTKLYWGSKAPTERGLSRKHIEGLKGLQR	180
rat K _v β 2	-----V-N-----IF-----A-E-146	146
bov K _v β 2	-----V-N-----IF-----A-E-146	146
rat K _v β 1	LQLEYVDVVFANRPDSNT ^o PHEEIVRAMTHVINQGHAMYG ^o SRWSAMEIMEAYSVARQFN	240
rat K _v β 2	-----P-----T-----S-----A-E-206	206
bov K _v β 2	-----P-----T-----S-----A-E-206	206
rat K _v β 1	MIPPVCEQAEYHLPREKVEVQLPELYHKIGVGAMTWSPLACGIIS ^o KYGVNGVPS ^o SRAS	300
rat K _v β 2	L--I-----M-----P-----V-----DS-I-PY-----	266
bov K _v β 2	L--I-----M-----P-----V-----DS-I-PY-----	266
rat K _v β 1	LKCYQWLKERIVSEGRKQNKLDLSPIAERLGLTLPQLAVAWCLN ^o EGVSVLLGSST	360
rat K _v β 2	-----G-----DK-L-----R-A-----E-QA-----I-----A-N-326	326
bov K _v β 2	-----G-----DK-L-----R-A-----E-QA-----I-----A-N-326	326
rat K _v β 1	PEQLIENLGAIQVLPMKTS ^o HVVNEIDNLRNKP ^o YSKKDYRS	401
rat K _v β 2	A--M--I-----LS-SI-H--S--G-----	367
bov K _v β 2	AD--M--I-----LS-SIH--S--G-----	367

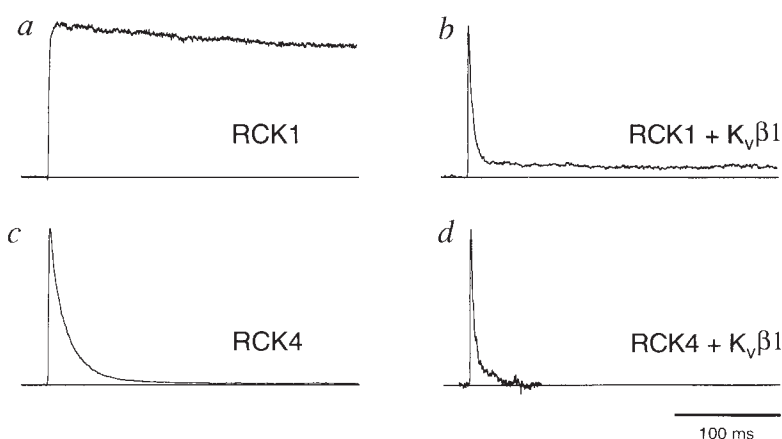
FIG. 1 Deduced protein sequences (single-letter amino-acid code) of cloned rat brain cDNAs encoding voltage-gated potassium channel β -subunits (K_v β 1 and K_v β 2). Sequences are compared to the β -subunit of bovine dendrotoxin receptor¹⁶ (bov K_v β 2). Identical amino acids are indicated by dashes. Amino-acid sequences are given for the longest open reading frames of K_v β 1 and K_v β 2 cDNA, respectively. Both cDNAs had in-frame stop codons upstream of the translation start codon ATG. Potential sites for phosphorylation by cAMP/cGMP-dependent protein kinase(s), and by protein kinase C or casein kinase II are marked by filled and open circles, respectively.

METHODS. Experiments with recombinant DNA followed standard techniques³⁶. 1×10^6 recombinants of a rat cortex λ gt10 cDNA library were hybridized at low stringency³⁷ with a ³²P-labelled fragment of bovine β -subunit cDNA (nucleotides 421–798). Fifteen cDNA clones were isolated for further analysis. K_v β 1 and K_v β 2 phage DNAs contained the longest inserts (1,706 and 1,700 nucleotides, respectively). After *Eco*RI digestion, phage DNA inserts were isolated and subcloned into pBluescript KS⁺. Both cDNA strands were sequenced (Sequenase kit 2.0, USB) and the sequence analysed using Genetics Computer Group Inc. software. cDNA sequences have been submitted to the EMBL data library (accession numbers X70662, X76724).

bound, pore-forming α - and the smaller β -subunits. It has been shown that DTX-receptor α -subunits correspond to members of the *Shaker*-related K_v1 (RCK)-channel family. DTX-receptor α - and β -subunits are tightly associated in a complex of 1:1 stoichiometry, indicating that RCK-type K_v channels may consist of $\alpha_4\beta_4$ heteromultimers *in vivo*¹⁵. The primary structure of a bovine DTX-receptor β -subunit has been determined¹⁶.

FIG. 2 Outward K^+ currents elicited by depolarizing pulses to +50 mV from a holding potential of -100 mV in the cell-attached patch-clamp recording configuration. Oocytes were injected with mRNA coding for a, $K_v1.1$ (RCK1); b, RCK1 + $K_v\beta1$; c, $K_v1.4$ (RCK4); and d, RCK4 + $K_v\beta1$. In both cases the β -subunit markedly accelerates inactivation. In some patches expressing RCK1 + $K_v\beta1$, the non-inactivating steady-state current component was significantly larger than that shown in d (up to 50% of the transient peak current).

METHODS. RCK1 (nucleotides 1–1,910), RCK4 (nt 1–2,658), $K_v\beta1$ (nt 1–1,546) and $K_v\beta2$ (nt 1–1,700) were subcloned into the expression vector pAKS2²³. The corresponding mRNAs were synthesized *in vitro* as described⁷. For each subunit, 12.5 ng mRNA was injected into oocytes from *Xenopus laevis*, prepared according to standard methods⁷. Patch-clamp recordings were performed 1–7 days after injection. Patch pipettes were made from aluminium borosilicate glass with resistances between 0.8 and 1.5 M Ω . The pipette solution contained 115 mM NaCl, 1.8 mM CaCl₂, 2.5 mM KCl, 10 mM HEPES, adjusted to pH 7.2 with NaOH. Currents were recorded with an EPC7 (List Medical, Darmstadt, Germany) or an EPC9 (HEKA Elektronik, Lambrecht, Germany) patch-clamp amplifier. The program package Pulse + PulseFit (HEKA Elektronik) was used for data acquisition and analysis. Leakage and capacitive currents



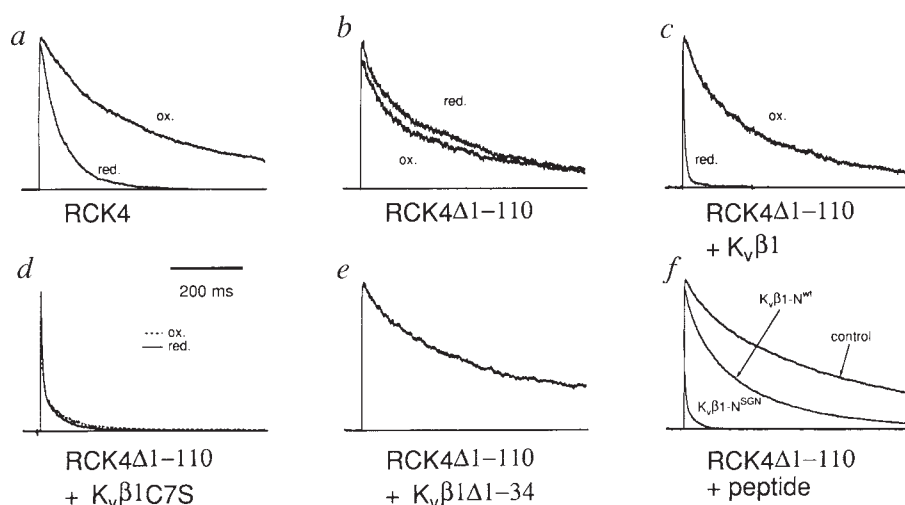
were subtracted on-line using a variable P/n subtraction method. Intervals between individual depolarizations ranged from 10 to 60 s depending on the speed of recovery from inactivation. Records were filtered with a low-pass Bessel characteristic of 1-kHz cut-off frequency. All experiments were done at room temperature. Note that injection of $K_v\beta1$ mRNA into *Xenopus* oocytes did not elicit outward currents.

Partial peptide sequence information from purified β -subunits was used to clone a bovine complementary DNA encoding a complete bovine $K_v\beta$ subunit.

Because rat K_v channels have been cloned and characterized more extensively than bovine K_v channels⁵, we cloned rat β -subunit cDNAs for functional characterization of $K_v\beta$ subunits. A ³²P-labelled probe of the bovine β -subunit cDNA was used to screen 1×10^6 recombinants of a λ gt10 rat cortex cDNA library. Fifteen independent cDNA clones were isolated. Sequence analysis of the cDNAs showed that these clones were derived from two different transcripts, $K_v\beta1$ and $K_v\beta2$, respectively. The longest open reading frame derived from a 1,700-base-pair (bp) $K_v\beta1$ cDNA sequence corresponds to a 401-amino-acid $K_v\beta1$ protein with calculated M_r 44.7K (Fig. 1). The longest $K_v\beta2$ open reading frame derived from a 1,700-bp $K_v\beta2$ cDNA sequence corresponds to a 367-amino-acid $K_v\beta2$ protein with calculated M_r

41K (Fig. 1). The rat $K_v\beta2$ subunit sequence shares 99% amino-acid identity with the previously obtained sequence for the bovine $K_v\beta$ subunit (bov $K_v\beta2$)¹⁶. The alignment of the bovine and rat $K_v\beta$ -protein sequences shows only four different amino acids (Fig. 1). Both proteins probably represent K_v channel subunits with similar functions in bovine and rat brain. By contrast, the rat $K_v\beta1$ protein sequence is markedly different particularly at its N terminus. The first 72 amino acids of rat $K_v\beta1$ do not align with the $K_v\beta2$ N terminus. The remaining 329-amino-acid C-terminal sequence is 85% identical to the corresponding rat and bovine $K_v\beta2$ sequences (Fig. 1). Most sequence differences are due to conservative amino-acid substitutions (Fig. 1). This result indicates that the β -subunits have a constant core region and a variable N terminus which may have a distinct function, as shown below. Analysis of the $K_v\beta$ subunit sequences for local hydrophobicity¹⁷ did not reveal the presence of typical mem-

FIG. 3 Inactivation mode of voltage-gated RCK4 channels is determined by the N terminus of $K_v\beta1$ subunits. Outward K^+ currents were elicited by depolarizing pulses to +50 mV from a holding potential of -100 mV in the inside-out patch-clamp recording configuration. a, RCK4; b, RCK4 Δ 1–110; c, RCK4 Δ 1–110 coexpressed with $K_v\beta1$; e, RCK4 Δ 1–110 coexpressed with $K_v\beta1$ C7S in oxidizing (ox., ~0.1% H₂O₂) or reducing (red., 5 mM glutathione) bath solution. d, RCK4 Δ 1–110 coexpressed with $K_v\beta1\Delta$ 1–34 lacking the 34 N-terminal amino acids. The remaining slow current decay is similar to that in a or c under oxidizing conditions and is attributed to the so-called C-type inactivation involving the channel pore structure^{38,39}. f, Currents recorded from inside-out patches expressing RCK4 Δ 1–110 before (control) and after application of 100 μ M peptide $K_v\beta1$ -N^{wt} and of peptide $K_v\beta1$ -N^{SGN}, respectively. The sequence of $K_v\beta1$ -N^{wt} corresponds to the first 24 amino acids of the $K_v\beta1$ amino terminus. In $K_v\beta1$ -N^{SGN}, the sequence GED (amino acids 17–19 of $K_v\beta1$) was substituted by SGN. **METHODS.** pAKS2-RCK4 Δ 1–110 was generated by digesting pAKS2-RCK4 (refs 7, 40) with *Pst*I, followed by religation. pAKS2- $K_v\beta1\Delta$ 1–34 was obtained by cloning a fragment of $K_v\beta1$ cDNA (nucleotides 437–780) generated by polymerase chain reaction (PCR) with an overhanging *Nde*I site and a *Hind*III site together with a *Hind*III/*Nde*I fragment of the 5' untranslated region of HBK2 cDNA (nt 453–863⁴¹) into *Hin* III-



digested pAKS2- $K_v\beta1$. pAKS2- $K_v\beta1$ C7S was generated by site-directed *in vitro* mutagenesis⁴². The identity of all clones used for mRNA synthesis was confirmed by restriction analysis and sequencing. The pipette solution in inside-out patches^{7,40} and analysis of mRNA expression in *Xenopus* oocytes are each described in Fig. 2 legend. The bath solution contained 115 mM KCl, 1.8 mM EGTA, 10 mM HEPES, adjusted to pH 7.2 with KOH. Peptides (Affinity Research, UK) were dissolved in the bath solution and applied to the inside-out patch by replacing the entire volume of the bath chamber.

brane-spanning segments or of a signal sequence. Potential *N*-glycosylation sites are also absent, consistent with the lack of evidence for attached carbohydrate on the native protein¹³. These structural properties indicate that $K_v\beta$ subunits are peripheral proteins which possibly associate with a cytoplasmic domain of $K_v\alpha$ subunits. A search through the protein data banks showed that $K_v\beta$ sequences have no apparent similarity to other protein sequences, including the β -subunits of voltage-gated sodium¹⁸ and calcium channels¹⁹. It therefore appears that β -subunits of voltage-gated ion channels are unrelated and do not belong to a gene superfamily like the pore-forming α -subunits⁸.

$K_v\beta 1$ and inactivation of K^+ channels

$K_v1\alpha$ and $K_v\beta 1$ subunits produce and form K_v channels with novel properties in expression systems *in vitro*. Cloned K_v1 (RCK) α -subunits by themselves form homo- as well as heterotetrameric K_v channels *in vitro*^{7,9,10} and possibly also *in vivo*²⁰⁻²². These channels mediate either slowly inactivating (delayed rectifier-type) or rapidly inactivating (A-type) potassium outward currents. Within the RCK family⁷, $K_v1.1$ (RCK1)²³ may be regarded as a typical representative of a delayed rectifier-type K_v channel and $K_v1.4$ (RCK4) as a typical A-type K_v channel⁷. Co-injection of RCK1 and $K_v\beta 1$ messenger RNAs in *Xenopus* oocytes elicited potassium currents that differed markedly from those obtained after injecting RCK1 mRNA alone (Fig. 2a, b). As previously reported²³, RCK1 mediates outward currents which decay by less than 20% within 10 s. In the presence of $K_v\beta 1$, however, RCK1 outward currents inactivate rapidly ($\tau_i = 4.7 \pm 0.4$ ms, mean $\pm$ s.d., $n = 4$). This suggests that the association of RCK1- α with $K_v\beta 1$ subunits converts RCK1 delayed rectifier-type inactivation into that characteristic of A-type currents. Likewise, co-injection of 1:1 weight ratios of RCK4 and $K_v\beta 1$ subunit mRNAs resulted in outward currents (Fig. 2c, d) which inactivated more rapidly ($\tau_i = 3.9 \pm 1.3$ ms, $n = 4$) than those mediated by RCK4 channels alone ($\tau_i = 26 \pm 10$ ms, $n = 5$). We have only tested the influence of $K_v\beta 1$ expression on two members of the RCK family. It is likely that the $K_v\beta 1$ subunit may also associate with other members of the RCK family converting them to A-type channels. By contrast, co-injection of RCK1 or RCK4 mRNA with $K_v\beta 2$ mRNA in *Xenopus* oocytes elicited macroscopic K^+ outward currents with inactivation modes similar to those expressed with RCK1 or RCK4 mRNA alone (data not shown). Therefore, $K_v\beta 2$ may have different functional properties.

A-type K_v channel α -subunits harbour within their N-terminal sequence an inactivating region that can block the internal mouth of the channel upon depolarization of the membrane^{24,25}. A ball-and-chain type mechanism has been proposed to explain this amino-terminal (N-type) inactivation of A-type K_v channels^{24,25}. The inactivating region of rat A-type K_v channel α -subunits (RCK4, Raw3) contains a cysteine that renders its N-type inactivation sensitive to oxidation^{26,27}. Inside-out patches of *Xenopus* oocytes containing RCK4 channels with or without $K_v\beta 1$ expressed A-type currents that were similarly sensitive to oxidizing conditions in the bath. Likewise, N-type inactivation was restored in both cases upon addition of the reducing agent glutathione (Fig. 3a). N-type inactivation is lost upon removal of the amino-terminal inactivating domain²⁴. Accordingly, RCK4 $\Delta 1$ -110 channels, which lack the N-terminal 'ball', mediate a slowly inactivating outward current (Fig. 3b); as expected, they are impervious to oxidation. But when RCK4 $\Delta 1$ -110 α -subunits are coexpressed with $K_v\beta 1$ subunits, a rapidly inactivating A-type current ($\tau_i = 4.2 \pm 0.8$ ms, $n = 8$) is observed under reducing conditions (Fig. 3c). Current traces were indistinguishable from those obtained for coexpressing RCK4 wild-type and $K_v\beta 1$ subunits (Fig. 2d). In an oxidizing environment, the rapidly inactivating current also disappeared (Fig. 3c). As previously shown, deletion of the N terminus (amino acids 6-46) in *ShakerB*²⁴ also removes rapid N-type inactivation of *Shaker*

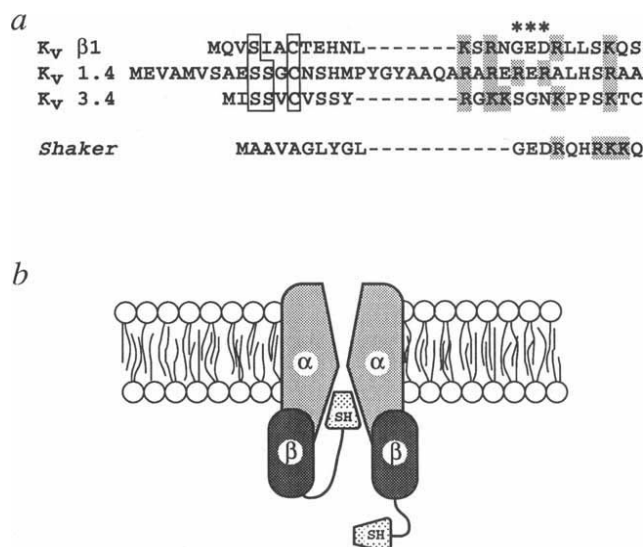
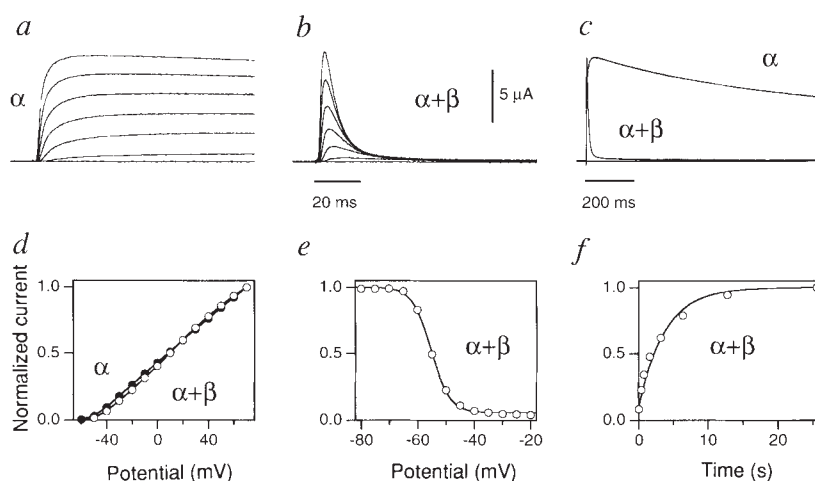


FIG. 4 a, Alignment of the N-terminal inactivating domain of $K_v\beta 1$ with those of α -subunits of voltage-gated K^+ channels. N-terminal sequences of α -subunits are from refs 7 ($K_v1.4$, RCK4), 43 ($K_v3.4$, Raw3) and 24 (*Shaker*). Gaps were introduced for optimal alignment and are indicated by dashes. Serines and cysteines in the serine/cysteine motif of rat α and β inactivating domains are framed. Arginines and lysines in the cluster of positively charged amino acids are shaded. For comparison, the *ShakerB*²⁴ inactivating domain is included in the alignment. This domain also contains a cluster of positively charged amino acids, but not a serine/cysteine motif. Asterisks denote the three amino acids (GED) that are replaced in peptide $K_v\beta 1$ -N^{SGN} by SGN to generate the peptide $K_v\beta 1$ -N^{SGN} (Fig. 3f). b, Model of the association of two α - and β -subunits of an $\alpha_4\beta_4$ K^+ channel¹⁵ showing the inactivating β -ball domains. The β -subunit binding site is proposed to associate with a cytoplasmic α -subunit domain. The inactivating β -ball domain can occlude the inner mouth of the open channel; we assume that one ball domain is sufficient for inactivation. SH refers to the sulphhydryl group of the cysteine that renders the inactivating ball domain sensitive to oxidation.

channels. When *ShakerB* $\Delta 6$ -46 mRNA²⁴ was coexpressed with $K_v\beta 1$ mRNA, rapid inactivation was restored (not shown), a result similar to that for RCK4 $\Delta 1$ -110 (Fig. 3c).

The simplest interpretation of these results is that $K_v\beta 1$ subunits themselves provide an inactivating ball domain with properties similar to those of K_v channel α -subunits^{24,26}. We therefore searched the $K_v\beta 1$ sequence for an inactivating ball domain (' β -ball') with structural similarity to the inactivation ' α -ball' of α -subunits. The alignment shown in Fig. 4a indicates that the $K_v\beta 1$ N terminus sequence is similar to the α -ball domain but is absent in $K_v\beta 2$. The rat α - and β -ball peptides both have a serine/cysteine motif followed by a cluster of positively charged amino acids (Fig. 4a), suggesting that the $K_v\beta 1$ N terminus could be responsible for the rapid inactivation of RCK1, RCK4 and RCK4 $\Delta 1$ -110 currents (Figs 2b, d and 3c). Accordingly, we constructed a $K_v\beta 1$ cDNA clone that expressed a $K_v\beta 1\Delta 1$ -34 subunit lacking the first 34 amino-terminal amino acids. Co-injection of RCK4 $\Delta 1$ -110 and $K_v\beta 1\Delta 1$ -34 mRNAs into *Xenopus* oocytes yielded outward currents with the expected properties: they did not inactivate rapidly and were insensitive to oxidation (Fig. 3d), like RCK4 $\Delta 1$ -110 channels (Fig. 3b), supporting the idea that the $K_v\beta 1$ amino terminus contains a β -ball. The sensitivity of RCK4 α -balls to oxidation is eliminated when the α -ball cysteine is substituted by serine²⁶. Replacement of the β -ball cysteine (position 7 in Fig. 1) by serine ($K_v\beta 1C7S$) had a similar effect on $K_v\beta 1$ -mediated K_v channel inactivation. Coexpression of RCK4 $\Delta 1$ -110 and $K_v\beta 1C7S$ mRNAs in *Xenopus* oocytes elicited rapidly inactivating currents, but the inactivation time constants were now indistinguishable under oxidizing or reducing conditions (Fig. 3e).

FIG. 5 Voltage-dependent activation and inactivation properties of A-type potassium currents elicited in *Xenopus* oocytes upon coexpression of RCK α and $K_v\beta 1$ subunits. Outward currents were recorded in the two-electrode voltage-clamp configuration. Holding potentials were -100 mV; depolarizing test potentials were from -60 to $+60$ mV in 20 -mV (a, b) or 10 -mV increments (d). Results are shown for RCK4 $\Delta 1$ –110 channels (a) to represent RCK α -subunits mediating slowly inactivating currents. Similar results were obtained with the α -subunits $K_v1.1$ (RCK1) and *Shaker*B46–46 upon coexpression with $K_v\beta 1$ (β). Scale bars indicate current amplitude and recording time. c, Current traces recorded after depolarizing pulses to $+60$ mV from a holding potential of -100 mV. Note that the time courses of rapid inactivation are similar for RCK4 $\Delta 1$ –110 + $K_v\beta 1$, $K_v1.1$ (RCK1) + $K_v\beta 1$ (Fig. 2b), and $K_v1.4$ (RCK4) + $K_v\beta 1$ (Fig. 2d). d, Current–voltage relationships for normalized currents mediated by α (filled circles) or by $\alpha + \beta$ (open circles). Peak current amplitudes were obtained from recordings like those in a and b. e, Steady-state inactivation of A-type outward currents observed at depolarizing pulses to $+20$ mV from different prepulse potentials (duration 1 s) varied from -80 to -20 mV in 10 -mV increments. f, Time course of recovery of A-type outward currents from inactivation. Currents were elicited from a holding potential of -100 mV by test pulses to $+50$ mV (pulse duration 50 ms), stepped back to the holding potential for various periods of time (interpulse interval) followed by a second test pulse.



The fraction of A-type current that had recovered between the first and second pulse was plotted versus the separating pulse duration.

METHODS. Recording and analysis of currents in the two-electrode voltage-clamp mode has been described⁷. The solid curve in e is a theoretical Boltzmann distribution based on a least-squares fit to the data. The curve in f describes a single-exponential recovery from inactivation, although two exponentials gave much better fits.

Compared with wild type, the fast-inactivating component is not complete because of an accelerated recovery from inactivation. These results suggest that serine substituted at Cys 7 destabilizes the complex between the $K_v\beta 1$ ball and the channel pore.

Like α -balls^{25,27}, covalent bonding of the $K_v\beta 1$ ball to the rest of the β -subunit is not necessary for its action. When $100 \mu\text{M}$ peptide corresponding to the $K_v\beta 1$ amino terminus ($K_v\beta 1\text{-N}^{\text{WT}}$) was applied to inside-out patches, inactivation of RCK4 $\Delta 1$ –110-mediated outward currents was accelerated (Fig. 3f). The inactivation time constant, however, was larger than that obtained after coexpression of RCK4 $\Delta 1$ –110 with full-length $K_v\beta 1$. Results with mutated *Shaker* α -balls^{24,25} indicate that an increase in positive charge may increase the on-rate constant for binding *Shaker* ball peptides to the channel pore. Consistent with this observation is the inactivating activity of the peptide $K_v\beta 1\text{-N}^{\text{SGN}}$, which has a more positive net charge than $K_v\beta 1\text{-N}^{\text{WT}}$ (Fig. 3f). Application of $100 \mu\text{M}$ $K_v\beta 1\text{-N}^{\text{SGN}}$ to inside-out patches containing RCK4 $\Delta 1$ –110 channels inactivated outward currents as fast as the $K_v\beta 1$ subunit itself (Fig. 3f). The results shown in Fig. 3 thus demonstrate that $K_v\beta 1$ subunits contain an inactivating ball in the amino-terminal sequence. Association of $K_v\beta 1$ with RCK α -subunits leads to the formation of A-type K_v channels, because the inactivating β -ball rapidly blocks the internal mouth of K_v channels upon depolarization (Fig. 4b).

We also investigated the activation and inactivation properties of the novel A-type currents obtained by coexpressing RCK- α and $K_v\beta 1$ subunits. The current–voltage relationship for delayed-rectifier-type outward currents mediated by RCK4 $\Delta 1$ –110 in the *Xenopus* oocyte expression system was not altered after their conversion into A-type currents by $K_v\beta 1$ subunits (Fig. 5a, b, d). Upon full channel activation (>0 mV), the time course of inactivation did not markedly depend on membrane potential (Fig. 5b). By contrast, the steady-state inactivation behaviour, measured by responses to test pulses to 50 mV from different prepulse potentials (prepulse duration of 1 s) showed a pronounced voltage-dependence (half-inactivation potential, $V_{1/2} = -59 \pm 4$ mV, slope factor = -2.8 ± 0.2 mV, $n = 10$). A-type currents elicited by RCK4 α -subunits alone (not shown) or by RCK4 α -subunits coexpressed with $K_v\beta 1$ (Fig. 5e) had a similar voltage-dependence of steady-state inactivation ($V_{1/2} = -54 \pm 5$ mV, slope = -3.3 ± 0.9 mV, $n = 6$). Inactivated A-type channels have a refractory period during which they recover

from inactivation. A protocol of two consecutive depolarizing test pulses interrupted by interpulse intervals of variable length was used to determine the time course of recovery from inactivation for an interpulse potential of -100 mV. The time constant for recovery from inactivation for A-type K_v channels mediated by RCK4 $\Delta 1$ –110 α - and $K_v\beta 1$ subunits (Fig. 5f) was 2.1 ± 1.1 s ($n = 7$). Recovery from inactivation was accelerated about eightfold (not shown), when the external K^+ concentration in the bathing solution was raised to 115 mM. As shown in Fig. 2, the presence of $K_v\beta 1$ accelerates the inactivation time course of RCK4 A-type currents. This is consistent with the finding that the time constant for the refractory period during which RCK4 channels recover from inactivation is increased about twofold by $K_v\beta 1$ (not shown).

$K_v\beta 1$ and $K_v1\alpha$ subunit RNA expression

Like RCK-type K^+ channel mRNAs²⁸, $K_v\beta 1$ mRNA occurs predominantly in brain tissue. Two $K_v\beta 1$ mRNA species of 3.6 and 3.9 kb were detected in northern blot experiments using total cellular rat RNA extracted from brain but not from heart, skeletal muscle or kidney (Fig. 6a). It is not clear whether or not these two $K_v\beta 1$ mRNA species represent splice variants. The expression of $K_v\beta 1$ mRNA was also studied by *in situ* hybridization to horizontal, frontal and parasagittal sections of rat brain. $K_v\beta 1$ mRNA is expressed most notably in the CA1/CA2 fields of the hippocampus and in caudate putamen (Fig. 6b, d). Some expression is also seen in dentate gyrus, in neocortical layers (pyramidal cells) (Fig. 6e), in the cerebellum (Purkinje cells) (Fig. 6f), and in thalamic nuclei. A comparison of $K_v\beta 1$ mRNA expression with that of RCK mRNAs²⁹ indicates that in different types of neurons $K_v\beta 1$ subunits might coassemble *in vivo* with different RCK α -subunits. For example, RCK1 mRNA is expressed in Purkinje cells, but not in corpus striatum²⁹, and RCK4 mRNA is expressed markedly in corpus striatum but not in Purkinje cells²⁹. Coexpression of RCK1 and $K_v\beta 1$ mRNAs may lead to the formation of RCK1/ $K_v\beta 1$ -type K^+ channels in Purkinje cells, whereas coexpression of RCK4 and $K_v\beta 1$ mRNAs may lead to RCK4/ $K_v\beta 1$ -type K^+ channels in corpus striatum. Distinct assemblies of α - and β -subunits in different neurons may thus contribute significantly to the structural and functional diversity of K_v channels in the mammalian nervous system.

Discussion

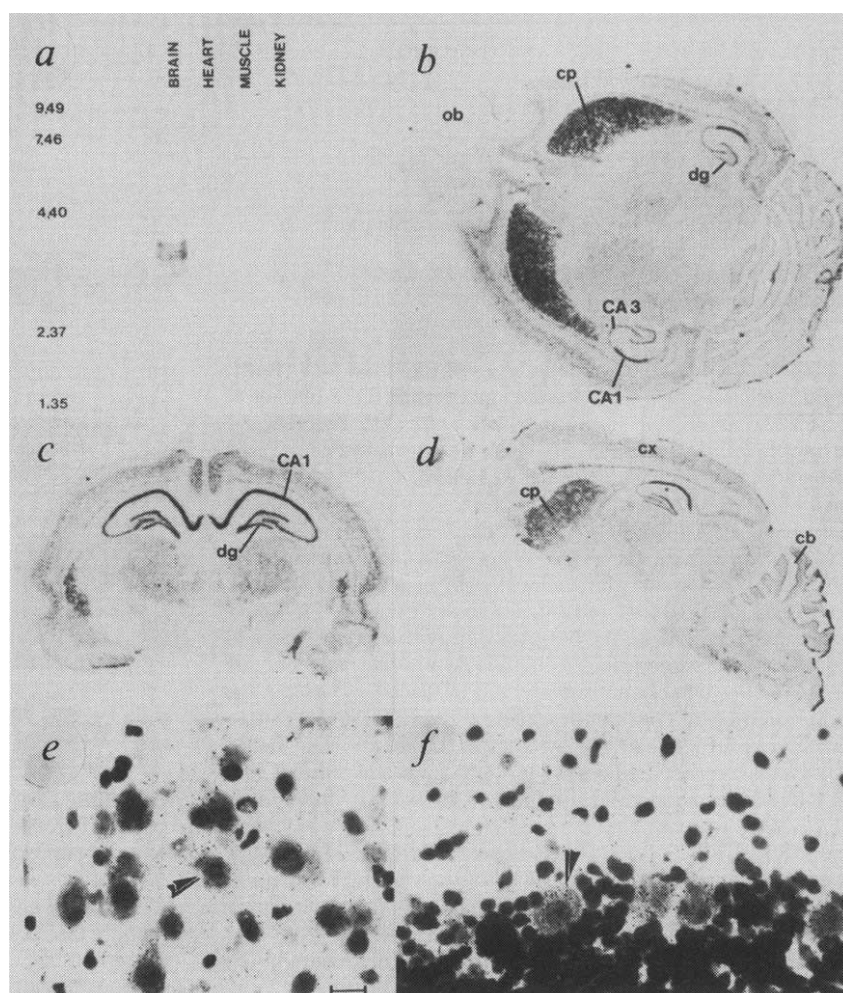
Low-molecular-mass rat brain poly(A)⁺ mRNA encodes factors that can slow³⁰ and accelerate³¹ outward currents mediated by A-type K_v channels. Some of these as-yet unidentified factors could be β -subunits: our results indicate that β -subunits are regulators of A-type K_v channel activity and, consequently, of neuronal output and firing patterns. A-type channel properties are conferred on delayed rectifier-type K_v channels through association with K_v β 1 subunits. The inactivating ball domain in the K_v β 1 amino terminus promotes rapid closure of open K_v channels which cannot otherwise inactivate rapidly. This mechanism implies that regulation of the activity of the β -subunit inactivation domain could determine the inactivation mode of K_v channels. We have shown that oxidation/reduction of a critical cysteine in the inactivation domain of K_v β 1 reversibly switches the channel inactivation mode from fast to slow and vice versa. As proposed for K_v channel α -balls²⁶, a fast and reversible formation of disulphide bridges between the inactivating ball domain and an as-yet unknown part of the K_v channel subunits may be the basis of the oxidation-sensitive change in inactivation mode. The conservation of this cysteine in three mammalian K_v-channel-inactivating domains (Fig. 4a) suggests that it is important biologically, for example as a potential sensor in oxidative stress in specific areas of mammalian neurons. An abnormal increase in oxygen radicals as in ischaemia³² would eliminate β -subunit-inactivating activity and thereby enhance K⁺ efflux.

A switch in gating mode of a voltage-gated cation channel in *Aplysia* bag-cell neurons triggered by a protein kinase A-regulated tyrosine phosphatase underlies the onset of a prolonged firing of spontaneous repetitive action potentials³³. Modulation of β -subunit activity by kinases may also lead to a switch in the inactivation mode of A-type K_v channels, leading to long-lasting changes in the firing frequencies of neurons by either increasing or attenuating nervous excitability.

The assembly of different hetero-multimeric protein complexes underlies the diversity of voltage-gated Na⁺ and Ca²⁺ channels in mammalian neurons³⁴. These channels are composed of a pore-forming subunit and up to four more subunits that are thought to have regulatory functions. Apparently, they mainly affect the rates of channel activation³⁵ and do not carry an inactivation domain like K_v β 1. The homo- as well as hetero-multimeric assembly of different pore-forming α -subunits^{5,7,9,10,20,22} contributes to the diversity of mammalian K_v channels. Our *in situ* hybridization data suggest that distinct combinations of α and β K_v channel mRNAs are expressed in different types of neurons. This is supported by the existence in bovine DTX receptors¹² of naturally occurring hetero-oligomeric K_v channels containing several $\alpha + \beta$ subunits²². Thus the formation of different combinations of α/β K_v channel subunits may increase the complexity and diversity of K_v channels. A corollary is that the behaviour of K_v channel α -subunits in expression systems *in vitro* may not faithfully reflect their properties *in vivo*. We

FIG. 6 Expression of K_v β 1-mRNA. **a**, Northern blot analysis of K_v β 1-mRNA. Rat tissue total cellular RNA (20 μ g each lane) was probed with a ³²P-labelled riboprobe transcribed from K_v β 1 cDNA in pBluescript SK⁺. Lanes were standardized for equal rRNA content. Positions of RNA size (kb) markers are indicated. **b**, **c** and **d**, Horizontal (**b**), frontal (**c**) and parasagittal (**d**) sections of P30 rat brain were hybridized with ³⁵S-end-labelled antisense oligonucleotides specific for K_v β 1. Strong signals were detected in the CA1/CA2 fields of the hippocampus and in the caudate putamen (cp); some signal intensity is seen in the neocortex (cx), the dentate gyrus (dg), in the CA3 fields of hippocampus, in thalamic nuclei (tn) and in the cerebellar Purkinje and granule cells. Cortical pyramidal (**e**) and cerebellar Purkinje cells (**f**) are shown at high magnification. Bar in **f** corresponds to 20 μ m. Note for comparison that RCK1 mRNA is not detected in the caudate putamen and is expressed prominently in CA3 but not in CA1 fields, and that RCK4 mRNA is not expressed in cerebellum²⁹.

METHODS. Northern blotting was done according to standard protocols³⁶. The hybridization probe was a ³²P-labelled riboprobe complementary to nucleotides 694–669 of K_v β 1 cDNA. Autoradiography was at –70 °C for 3 days with an intensifying screen (DuPont). RNA size markers were from Gibco BRL. *In situ* hybridization has been described²⁹. Two different antisense oligonucleotides (CACCGCTACCCACAGGATTTACTCATGCAGCTTAAGTGCTCATGCAT, complementary to the 3' untranslated K_v β 1 cDNA sequence (nt 1,591–1,542); and TGCACAGTGGTAGCAACAGCAGAGACCTCAGAGAATCCTGGGACACACTAGA, complementary to the 3' untranslated K_v β 1 cDNA sequence (nt 1,696–1,647) were used. Hybridizations in **b**, **c**, **d**, **e** and **f** were done with the first antisense oligonucleotide, the second was used for independent confirmation. A sense oligonucleotide complementary to the first antisense oligonucleotide was used as a negative control (not shown).



propose that most cloned α -subunits^{5,6}, which form slowly inactivating K_v channels *in vitro*, can acquire A-type K_v

channel properties if they are assembled with a β -subunit like $K_v\beta 1$. □

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LETTERS TO NATURE

Estimate of the intergalactic infrared radiation field from γ -ray observations of the galaxy Mrk421

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THE magnitude of the intergalactic infrared radiation field (IIRF) is of fundamental importance to investigations of the evolution of galaxies. Bursts of star formation, which seem to be critically important to galaxy evolution, are characterized by large infrared luminosities¹, yet we have little knowledge of the frequency and intensity of starbursts in the early Universe. Unfortunately, direct observational determinations of the IIRF are plagued by the difficulty of separating the extragalactic emission from Galactic foreground radiation and the zodiacal light in the Solar System, and have so far produced only upper limits that are far above theoretical expectations². We have previously proposed³ a way to use ground-based observations of high-energy γ -rays to probe the IIRF, and here we apply our method to the spectrum^{4,5} of the BL Lac object Mrk421. The intensity we derive for the IIRF is consistent with the conversion of at least 30% of the energy from stellar nucleosynthesis to infrared radiation, both from emission by cool stars and as a result of absorption and re-emission by interstellar dust grains.

The IIRF can be probed by looking for absorption of very high-energy γ -rays in the spectra of blazars³. The absorption process involves the collision of a γ -ray photon from the blazar with a photon of the IIRF. In the collision, both the γ -ray and the infrared photon disappear and their energy reappears in the form of an electron–positron pair. Observations of high-energy (GeV) γ -rays from blazars have been made by the EGRET (Energetic Gamma-Ray Experiment Telescope) detector on the Compton Gamma Ray Observatory satellite. Observations of

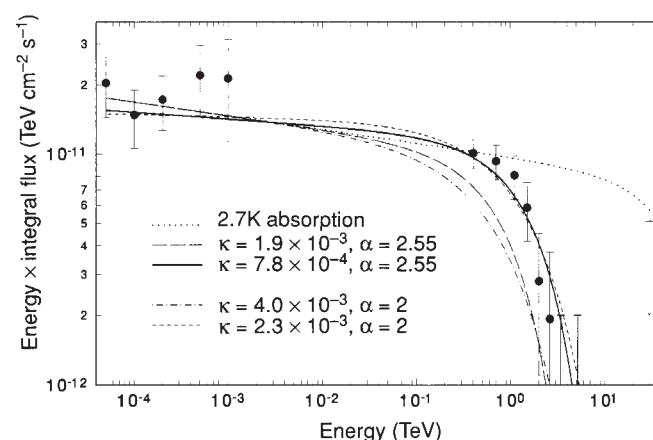


FIG. 1 The Mrk421 integral γ -ray energy spectrum from EGRET⁵ and Whipple⁴ observations (filled circles) compared to theoretical spectra corresponding to 2.7 K absorption only (dotted line) and absorption expected from the (2σ) upper limits of ref. 10 (long dashed and dot-dash lines). The solid line ($\kappa = 8 \times 10^{-4}$) represents our measured spectrum corresponding to $\alpha = 2.55$. Intergalactic electron–photon cascading^{17,18}, which would result in a pile-up towards lower energies, is not expected to influence the TeV spectrum of Mrk421, because the source is observed within a field-of-view of $\Delta\theta \approx 10$ arcmin (ref. 4), and it can be shown that an intergalactic magnetic field of $B \approx 3 \times 10^{-19} (E_e/10 \text{ TeV}) (\Delta\theta/10^\circ) \text{ G}$ or greater would deflect the cascade TeV electrons (with energy $E_e = 10 \text{ TeV}$) away from the source direction. As the measured magnetic field strengths in superclusters¹⁹ are of the order of 10^{-6} G , it is highly unlikely that the intergalactic field would be as weak as $\sim 10^{-19} \text{ G}$. The Tibet air shower upper limit²⁰ is shown at 30 TeV.

very high-energy (TeV) γ -rays can be made by using ground-based Cherenkov detectors to observe the light from γ -ray-induced atmospheric air showers⁶. We have recently used such observations to place conservative upper limits on the IIRF and rule out exotic mechanisms for its production⁷. Here, we attempt to go further, interpreting the observed dearth of photons above 3.4 TeV in the Whipple Observatory ground-based data as most reasonably being *prima facie* evidence for absorption by the IIRF and thus providing a first estimate of its magnitude.

We have previously stated that simultaneous satellite and ground-based observations would generally be desirable in order

to determine accurately the spectral characteristics of a source³. However, in the case of Mrk421, simultaneous observations do not appear to be required. Although the Whipple⁴ and EGRET⁵ observations were not simultaneous, averaging over the observing period of several weeks were needed to obtain the Whipple data, resulting in at most a 20% variation in the Mrk421 flux between 1992 and 1993 (ref. 8). Also, the EGRET observations have shown no evidence of changes in the spectral index of blazar sources for which the total flux is known to be highly variable. Both of these points, together with the excellent fit of the data to a single power-law over the whole energy range from 70 MeV to TeV energies, give us confidence that, in this case, we have a good spectral determination.

The differential energy spectrum of Mrk421, obtained from both EGRET and Whipple observations^{4,5}, has an intensity given by⁷

$$I(E) = (1.02 \pm 0.14) \times 10^{-11} E^{-2.06 \pm 0.04} \text{ photon cm}^{-2} \text{ s}^{-1} \text{ TeV}^{-1} \quad (1)$$

up to an energy E of ~ 3 TeV. In our previous analysis⁷, the spectral information above 3.4 TeV was neglected; here, it is just these data that play a key role in our determination of the IIRF. Using Table 1 of ref. 4, and extrapolating equation (1), we expect ~ 58 events when looking directly at the source, 'on-source' (source γ -rays and cosmic-ray background), above 3.4 TeV, whereas only 26 on-source events were observed, consistent with the 27 off-source (looking away from the source) cosmic ray background events collected with similar exposure. Furthermore, above 5.1 TeV we expect ~ 32 on-source events, whereas only 10 are observed against the 12 background events. The significance of this lack of events from this source is $\sim 4\sigma$ (standard deviations) when taking the uncertainty in the spectral index into account with a χ^2 fit.

We will interpret these data as being the result of absorption by the IIRF. Consider the interaction of a γ -ray of energy E with a soft photon of energy ϵ . Pair production is expected above the threshold energy condition for generating an electron and a positron, each of mass m , $E\epsilon(1 - \cos \theta) > 2(mc^2)^2$, where θ is the angle between the photon directions.

The collision cross-section σ for the pair production absorption process, involving a γ -ray of energy E and an infrared photon of energy ϵ , is maximized when

$$\epsilon \approx \frac{2(mc^2)^2}{E} \approx 0.5 \left(\frac{1 \text{ TeV}}{E} \right) \text{ eV} \quad (2)$$

so that a 1-TeV γ -ray is most effectively absorbed by a K-band infrared photon of energy $\sim 1/2$ eV and wavelength $\sim 2 \mu\text{m}$.

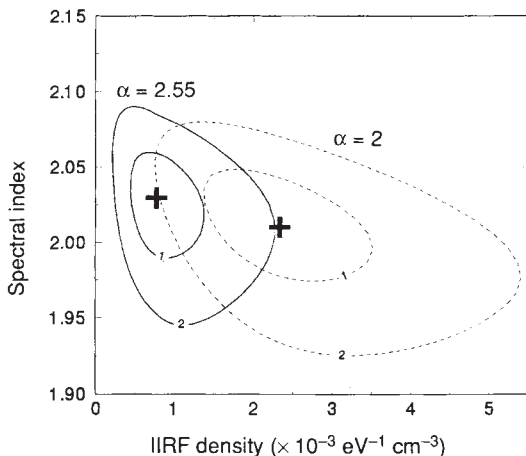


FIG. 2 Two-dimensional contour plot of spectral index Γ versus the IIRF density κ for $\alpha = 2.55$ (solid contours) and $\alpha = 2$ (dashed contours). The cross indicates the best solution for each α (χ^2 per degree of freedom < 1 in both cases) and the contours are marked by '1' and '2', indicating 1σ and 2σ confidence contours respectively.

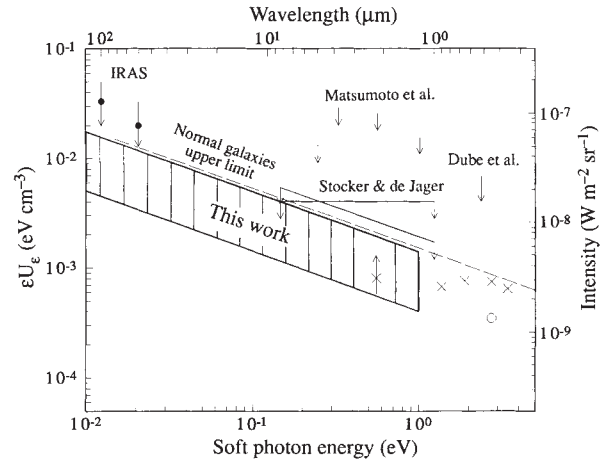


FIG. 3 The extragalactic infrared background photon density (ϵU_ϵ) versus soft photon energy (ϵ) (after Fig. 1 of ref. 7). Intensity and wavelength units are also shown. The spectrum corresponding to the maximum contribution from galaxies⁴ is shown as a long dashed line labelled 'Normal galaxies upper limit'. The upper limits derived from direct observation in the optical (Dube *et al.*²¹) and the near infrared (Matsumoto *et al.*²²) are shown as indicated by arrows. The model estimates by Tyson²³ are shown as crosses and that of Yoshii and Takahara²⁴ by an open circle. The previous upper limits from TeV observations⁹ for $\alpha = 2$ and $\alpha = 2.55$ are indicated as 'Stocker & de Jager'. Our present possible detection is shown as a slanted box labelled 'This work'.

If the IIRF photon density is n , and the distance to the source is given by $L = cz/H_0$, (where H_0 is taken to be $75 \text{ km s}^{-1} \text{ Mpc}^{-1}$ and $z = 0.031$ for Mrk421), the optical depth for a γ -ray will be roughly $\tau \approx n\sigma L$. The differential energy spectrum $I(E)$ for Mrk421 given by equation (1) is assumed to hold true at the source for all energies of interest. We then expect an absorbed differential spectrum of the form $I(E) \exp(-\tau(E))$. We therefore determine the IIRF density n by determining $\tau(E)$ from the spectral data, yielding $n \approx \tau/\sigma L$. For a more exact and detailed description of the calculations, see refs 3 and 7.

The combined EGRET and Whipple spectral data points were used to construct the integral spectrum shown in Fig. 1. From a χ^2 -fit, we obtained the best-fit spectra (and hence the parameters for the IIRF) by comparing the observed flux values $F_1, \dots, F_{13}$ with the expected flux values obtained using a differential γ -ray spectrum of the form

$$I(E) = K E^{-\Gamma} \exp -\tau(E; \kappa, \alpha) \quad (3)$$

The parametric expression which we used for the optical depth is $\tau(E; \kappa, \alpha) = a(\alpha)\kappa E^{a-1}$, which was derived using power-law spectra for the IIRF of the form $n(\epsilon) = \kappa \epsilon^{-\alpha}$ with the two values for α of 2.0 and 2.55 taken for a galaxy generated IIRF⁹. For $\alpha = 2$ we find $a = 120$, whereas $a = 143$ for $\alpha = 2.55$. The free parameters of the χ^2 -fit are K , Γ and κ , given a value for α . The details of the fits are given in the captions of Fig. 1. The best-fit integral spectra for $\alpha = 2$, and $\alpha = 2.55$, are shown in Fig. 1 by the short dashed line and thick solid line respectively.

Because the errors on the spectral index Γ and the IIRF constant (κ) are correlated, we constructed the 1σ and 2σ (standard deviations) confidence contours for $\alpha = 2$ (short dashed contours) and 2.55 (solid contours) as shown in Fig. 2. The confidence contours indicate the range for κ , given the uncertainty in Γ . The sharpness of the cutoff seen in Fig. 1 indicates that $\alpha = 2.55$ is marginally preferred relative to $\alpha = 2$, and for $\alpha = 2.55$ we obtain the best-fit spectrum of

$$n(\epsilon) \approx (0.8_{-0.4}^{+0.6}) \times 10^{-3} \epsilon^{-2.6} \times \left(\frac{H_0}{75 \text{ km s}^{-1} \text{ Mpc}^{-1}} \right) \text{ cm}^{-3} \text{ eV}^{-1} \quad (4)$$

for $0.01 \text{ eV} \leq \epsilon \leq 1 \text{ eV}$

This range is shown by the slanted box in Fig. 3.

There are three main caveats which are related to our analysis. (1) There may be some uncertainty in determining the event rate derived by Whipple *et al.* for their highest energy events owing to a possible oversubtraction of assumed proton-produced events, which can become more confused with γ -ray-produced events at higher energies. We note, however, that the Crab nebula was observed by the Whipple group to have a power-law spectrum with no rollover reported up to 10 TeV. The Whipple spectra of the Crab nebula and Mrk421 both connect smoothly with the EGRET spectra^{4,10}, suggesting that other systematic effects may not be too much of a problem in the analysis of the Whipple data. (2) The source spectrum may have an intrinsic turnover above ~ 3 TeV. But one may then ask why the turnover would occur at exactly the energy expected for an IIRF absorption effect. We also note that emission models involving Compton scattering of high-energy electrons in the source would not produce such a sharp cutoff^{11–13}. (3) Intrinsic absorption in the source may cause the high-energy cutoff. However, we consider it improbable that the infrared characteristics of the source would conspire to exactly mimic that which would be produced by a reasonably estimated IIRF. (Note that if the absorption is intrinsic, our estimate becomes at best an upper limit.)

Therefore, we conclude that our result may indeed be the first determination of the IIRF at a level which is expected from theoretical arguments^{3,9}. Our roughly derived IIRF spectrum suggests that the IIRF arises from stellar processes in galaxies⁷ and that, on average, at least 30% of the total energy released in stellar nucleosynthesis (including that from infrared and starburst galaxies) eventually ends up in the infrared wavelength band. Finally, we note that if the IIRF, and thus n can be directly measured, as may eventually be possible with the DIRBE

(Diffuse Infrared Background Experiment) on the COBE satellite¹⁵, we may be able to obtain a new measurement of the Hubble constant $H_0 \propto n/\tau$ at cosmologically significant distances¹⁶. $\square$

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Detection of two interstellar absorption bands coincident with spectral features of C_{60}^+

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MORE than a hundred well-defined absorption bands, arising from diffuse gas in the interstellar medium, have been observed in the visible and near-infrared spectra of stars^{1–4}. The identity of the species responsible for these bands has remained unclear, although many possibilities have been suggested^{5,6}. Carbon-based molecules ubiquitous in the interstellar medium have been widely favoured as potential carriers of some of the diffuse interstellar bands^{7–10,29}; in particular, C_{60}^+ has been thought to be a promising candidate^{9,29}. Here we present the results of a search for C_{60}^+ in the near-infrared spectra of seven stars, based on recent laboratory measurements of the absorption spectrum of this species^{11–13}. We find two diffuse bands that are coincident (within 0.1%) with laboratory measurements on C_{60}^+ in a Ne matrix¹¹. From this observation and the total absorption, we estimate that 0.3–0.9% of interstellar carbon is in the form of C_{60}^+ . The molecule is very stable, which should allow it to survive in the interstellar medium for a long time¹⁴, but the inhibition of C_{60} formation by hydrogen probably limits its abundance.

The C_{60} molecule has attracted much attention since Kratschmer *et al.*¹⁵ succeeded in synthesizing it in macroscopic quantities by vaporizing graphite in a He atmosphere. C_{60} is expected to be the most stable and dominant fullerene during clustering¹⁴, but the visible absorption spectrum of neutral C_{60}

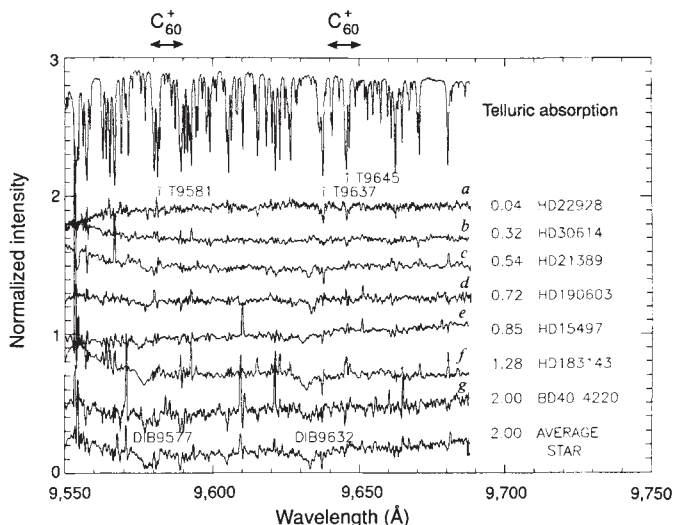


FIG. 1 a–g, Spectra of the seven stars studied, in order of increasing reddening. To the right of each trace are shown the associated $E(B-V)$ value, and the star name. Each observed star spectrum has been, after instrumental corrections, divided by a spectrum of a reference star (of similar spectral type) observed immediately at the same airmass. This divided out both the telluric water absorption bands and the stellar lines. The top trace is a reference spectrum of η Ursae Majoris (with offset of 2), showing the strength of the telluric water bands. Slight residuals from telluric corrections are indicated (T). Two new diffuse interstellar bands (DIBs) are detected at 9,577 and 9,632 Å, increasing with $E(B-V)$. The slight wavelength shifts, up to 1.3 Å, and different line profiles are associated with the velocity distribution of interstellar clouds along the line of sight. The bottom trace is an average composite spectrum corresponding to $E(B-V) = 2$. We also give the positions of the two C_{60}^+ main bands measured in Ne matrix by Fulara *et al.*¹¹.

does not show any match with current observations of diffuse interstellar bands (DIBs)¹⁵. However, the first electronic transitions in many cations or radical cations of polycyclic aromatic hydrocarbons (PAHs) and fullerenes are expected in the near-infrared¹⁶. In 1992, the electronic absorption spectrum of the C_{60} radical cation (readily distinguished from the anion by the use of an electron scavenger) was determined in solid matrices of freon¹² and argon¹³. The results show a general agreement for band positions, unresolved at 9,800 Å in freon, but with two peaks (with respective widths 100 and 40 Å) in argon, that could be resolved at 9,676 and 9,728 Å using magnetic circular dichroism¹³. These data agree fairly well both with the value calculated (8.95–7.6 eV) from the photoelectron spectrum¹⁷ of C_{60} , and with a recent spectrum in a Ne matrix¹¹.

We searched for the C_{60}^+ cation by observing in the near-infrared range near these expected positions. Here we report the detection of two strong new DIBs consistent with a C_{60}^+ origin. The data were obtained with the 1.52-m Coudé telescope, Aurélie spectrograph and 2,048 diode-array detector at the Observatoire de Haute Provence (OHP), France. We observed first in September 1992 at medium resolving power $R=9,000$, in the range 9,300–10,000 Å where we detected two dominant lines at 9,577 and 9,632 Å. However, the spectral domain at 9,600 Å is strongly absorbed by the Earth's atmospheric water (Fig. 1, top trace). We therefore decided to improve the sensitivity, resolution and observing strategy on 15–17 February 1993 and 12 November 1993, using a spectral resolving power of $R=36,000$ over the range from 9,500–9,700 Å. The stars we examined (hot spectral types from O7 to A0 to limit stellar photospheric lines) were selected to cover a range of interstellar reddening $E_{(B-V)}$ from 0 to 2 mag. Observing in this region requires a detailed telluric correction by immediately observing, at the same airmass, unreddened reference stars of similar spectral type and rotation velocity. This allows the simultaneous removal of stellar photospheric lines. The spectra were reduced and analysed at OHP and at ESA Solar System Division. Figure 1a–g shows a series of the telluric-corrected spectra for the measured stars. The two new diffuse bands at 9,577 and 9,632 Å were confirmed, showing increasing strength with reddening and no dependence on spectral type. From a composite spectrum (Fig. 1, lowest trace) combining the five more reddened stars and scaled to $E_{(B-V)}=2$ mag, we limit the telluric residuals and determine that the far wings extend up to 4 Å from the centre of the bands. Possible interfering stellar transitions of Mg II (9,631.888 and 9,632.435 Å) are corrected well by the reference star spectrum division, as they did not appear in the reduced spectra of the less reddened stars. Also, the widths of the 9,632 Å band (full width at half maximum 2.4–4 Å for different lines of sight) are much larger than the stellar rotational broadening measured in the slow rotating stars such as HD21389 or HD190603. Figure 2 shows, for the seven stars studied, the good correlation (correlation coefficients 0.91 and 0.86) between the equivalent widths of the two new diffuse bands and the reddening, $E_{(B-V)}$, with slopes of 0.39 and 0.67 Å per unit $E_{(B-V)}$, confirming the detected bands as new DIBs. The even tighter correlation between the bands themselves (coefficient 0.98) with a ratio of 1.6 ± 0.15 suggests a similar or a single carrier.

Recently the spectrum of C_{60}^+ has been measured both in argon^{11,13} and neon (ref. 11, and L. d'Hendecourt, K. Fostiropoulos and A. Léger, unpublished results¹⁸) matrices. Neon is known to provide the best optical matrix (with minimum scattering) and also introduces minimum shift and broadening of bands with respect to the gas phase¹⁹. The two dominant C_{60}^+ spectrum peaks are at $9,663 \pm 9$ and $9,724 \pm 4$ Å in Ar, and at $9,580 \pm 4$ and $9,642 \pm 3$ Å in neon¹¹; typical matrix-broadened widths are 40 Å and the bands have an intensity ratio of 1:1.5. Because the two cation bands appear in both matrices with the same average separation (61 ± 10 and 62 ± 6 Å), they are probably intrinsic to two major ground-state geometries favoured by Jahn-Teller types of distortion, and not a matrix-splitting effect

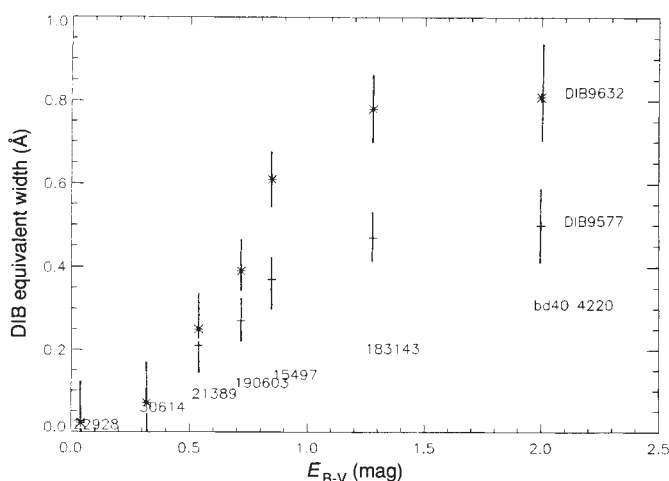


FIG. 2 Plot of the equivalent widths of the newly detected DIBs at 9,577 and 9,632 Å (shown as crosses and stars respectively) as a function of reddening $E_{(B-V)}$ (the slopes are 0.39 and 0.67 per unit $E_{(B-V)}$, and the correlation coefficients are 0.91 and 0.86 respectively). Under each pair of points is shown the identifying number of the relevant star. Values for HD22928 and HD30614 represent upper limits. Typical errors on the equivalent width are 0.05 Å due to photon noise and telluric correction: $\pm 2\sigma$ error bars are given for each measurement. The departure of BD40 4220 from the relation is also observed in the 5,780 Å DIB¹, suggesting a specific effect along this line of sight. The tighter correlation between the bands ($R=0.98$) supports the case for a single carrier, with DIB positions and relative strength consistent with the C_{60}^+ laboratory spectra.

because of different kinds of sites. C_{60}^+ shows no other absorption bands in the 5,000–10,000 Å range with an extinction stronger than 20% of the 9,577/9,632 Å bands^{11,12}.

We measure consistent shifts between the C_{60}^+ laboratory peaks and our detected interstellar bands at 9,577 and 9,632 Å. The band positions of C_{60}^+ taken in an argon matrix (average shift of 86 and 92 Å) converge in a neon matrix (shift 3 and 10 Å) towards the proposed DIBs (within 0.1% of the cited wavelength), without overshooting this position as discussed for the pyrene cation²⁰ (considered as a potential DIB carrier). The systematic laboratory redshifts and broadening can be explained by known matrix effects^{20–23}. The positions and relative strength (ratio 1:1.6) of the two new DIBs, dominant in this range, are thus consistent with the two main bands measured in laboratory C_{60}^+ spectra. This is strong evidence for the existence of the C_{60}^+ cation, as the largest molecule detected in interstellar space.

Due to large number of configurations and symmetry problems associated with open shells, quantum-mechanical calculations for the C_{60} ions are far more complicated than for neutral C_{60} , and yield only approximate transitions positions and oscillator strengths^{24,25}. Bendale *et al.*²⁵ find that the ground state of C_{60}^+ is distorted from the I_h optimized symmetry of neutral C_{60} , and the D_{5d} geometry is the preferred static equilibrium structure with a Jahn-Teller distortion stabilization energy of 8.1 kcal mol⁻¹. At temperatures encountered in the interstellar medium, this is probably the ground state prevailing in the absence of interaction or excitation. In the preferred D_{5d} geometry, degenerate transitions ($E_{1g} \rightarrow A_{1u}$) are predicted at 8,630 cm⁻¹ with total oscillator strength 0.14, whereas in the I_h geometry, two transitions ($H_g \rightarrow H_u$ and $G_g \rightarrow H_u$) are calculated at 8,600 and 8,900 cm⁻¹ with same oscillator strength 0.047. Due to the combination of wavefunctions in these limit geometries, two close and dominant electronic transitions are expected, as seen in the laboratory. Kato *et al.*¹² measured absorption spectra of the C_{60} cation and anion. Considering their experimental

uncertainties, and scaling from the published spectra using the quoted extinction for the C_{60} radical anion transition, we derive a lower limit of 0.004–0.012 for the total oscillator strength of the two C_{60}^+ transitions at 9,600 Å. But because the observed 0–0 transition in the near-infrared is a dipole-allowed transition, the oscillator strength could be as high as 1. We derive here a probable C_{60}^+ interstellar abundance that can easily be refined when an accurate oscillator strength of the transition is available.

For small PAH ions, such as the naphthalene cation and pyrene cation, abundances of 0.3% and 0.2% of cosmic carbon have been estimated (the oscillator strength for the pyrene cation, which shows a transition very near the dominant DIB at 4,430 Å, was measured in an Ar matrix to be 0.126)^{20, 22}. Based on these limits, we assume an oscillator strength for C_{60}^+ of 0.1 (also consistent with theoretical estimates²⁵), and we estimate the abundance with the Spitzer formula²⁶. Using the total equivalent width ΔW_λ of 1.06 Å per unit reddening $E_{(B-V)}$ derived from our 9,577/9,632 Å observations, assuming a ratio $C/H = 3.7 \times 10^{-4}$ and hydrogen column density per unit interstellar absorption $N_H/A_V = 2 \times 10^{21} \text{ cm}^{-2}$ (with $A_V = 3 E_{(B-V)}$), we calculate that 0.035% of the cosmic carbon could be in the form of C_{60}^+ (taking the lower oscillator strength limit derived from Kato *et al.*¹², this becomes at maximum 0.3–0.9% of the cosmic carbon). Even for the lower abundance limit (using the theoretical oscillator strength), C_{60}^+ could play a significant role in the interstellar chemistry.

The spontaneous formation of C_{60} together with carbon dust (especially in regions depleted in H), and the stability of its polyhedral structure against intense radiation, ionization and chemical attacks, control the survival of this molecule¹⁴. The low ionization potential of 7.61 eV will favour the ionization of C_{60} in diffuse interstellar medium outside dark clouds (which is the place of residence of the carriers of the DIBs) but less in ultraviolet-shielded molecular clouds²⁹. The DIB carriers are probably created in circumstellar environments and their formation must be therefore strongly influenced by circumstellar abundances and physical processes²⁷. Obviously one does not expect prominent DIB features in those circumstellar environments where progenitors of ionized DIB carriers are in a different state. The variations of environment (abundance, ultraviolet photoionization) must be manifested in anomalies of band intensities or even in absence of specific DIBs^{27, 28}. The inhibition by hydrogen of the fullerene growth mechanism will limit its formation to very specific H-depleted environments, and may explain its relative abundance compared to PAH ions. But the stability of C_{60} allows it to survive long enough to be cycled into the diffuse interstellar medium, and thus be detected where ionized (with limited line-of-sight abundance variations) as found in this Letter. Alternatively, our estimate for the C_{60}^+ abundance can be considered as only the 'immaculate' contribution, as other fullerenes may exist as stable exohedral or endohedral metallo-complexes¹⁰, which would give rise to other DIBs.

We hope that the results reported here will provide a stimulus to laboratory spectroscopists, chemists and physicists in the study of fullerenes, PAHs, and their ions and complexes. Astronomers need not only accurate band positions but also calibrated band intensities and gas-phase line profiles in order to search for and identify other potential DIB carriers, and to derive their abundances or physico-chemical properties. □

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Synthesis of polymeric microcapsule arrays and their use for enzyme immobilization

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CURRENT methods for immobilizing enzymes for use in bioreactors and biosensors^{1–20} include adsorption on or covalent attachment to a support^{2–4}, micro-encapsulation^{5,6}, and entrapment within a membrane/film^{7,8,11–20} or gel⁹. The ideal immobilization method should employ mild chemical conditions, allow for large quantities of enzyme to be immobilized, provide a large surface area for enzyme–substrate contact within a small total volume, minimize barriers to mass transport of substrate and product, and provide a chemically and mechanically robust system. Here we describe a method for enzyme immobilization that satisfies all of these criteria. We have developed a template-based synthetic method that yields hollow polymeric microcapsules of uniform diameter and length. These microcapsules are arranged in a high-density array in which the individual capsules protrude from a surface like the bristles of a brush. We have developed procedures for filling these microcapsules with high concentrations of enzymes. The enzyme-loaded microcapsule arrays function as enzymatic bioreactors in both aqueous solution and organic solvents.

The synthetic methods developed are related to our work on polymeric microtubules^{21–24}. Although such tubules^{21–27} are interesting microstructures, they are of doubtful utility for enzyme immobilization because they are microscopic cylinders that are open at both ends. The synthetic strategies described here yield capped versions of such microtubules (that is, 'microcapsules'). Microporous polycarbonate filtration membranes^{21–24} were used to prepare these microcapsules. These membranes have cylindrical pores of uniform ($\pm 10\%$) diameter; these pores are used as templates for preparation of the microcapsules. The membranes used here contained 1×10^8 pores cm^{-2} , and the pores were 400 nm in diameter. Membranes with significantly higher porosities and with pore diameters as small as 10 nm are available. Hence microcapsule arrays of the type described here can be prepared with almost any desired capsule density and diameter. Finally, the length of the microcapsules

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obtained is determined by the thickness of the template membrane, in this case 10 μm .

The microcapsule arrays are prepared via a combination of electrochemical and chemical polymerizations. The surface of the template membrane is first sputtered with a $\sim 50\text{-nm}$ layer of gold (Fig. 1a), which is used to electropolymerize²⁸ a polypyrrole film across the face of the membrane. Short (1 μm) polypyrrole 'plugs' are also deposited within the pores (Fig. 1b). Polypyrrole microtubules are then chemically polymerized²¹⁻²⁴ within the pores of the plugged membrane (Fig. 1c). The electrochemically polymerized plugs become caps for the chemically polymerized tubules.

The capped microtubules are then filled with the desired enzyme by vacuum filtration of a solution of the enzyme through the capsule-containing membrane (Fig. 1d). The solvent molecules (H_2O) can pervaporate through the polypyrrole plugs, whereas the much larger enzyme molecules are retained within the capsules. Five enzymes—glucose oxidase, catalase, subtilisin, trypsin and alcohol dehydrogenase—have been encapsulated and tested so far. After addition of the enzyme, Torrseal epoxy is applied to the upper surface of the membrane (Fig. 1e). After curing, the entire assembly is immersed in methylene chloride to dissolve the membrane. This yields the desired array of enzyme-loaded microcapsules (Figs 1f and 2a). A transmission electron micrograph of capsules that have not been attached to the epoxy surface is shown in Fig. 2b. The walls of the capsules are so thin ($\sim 25\text{ nm}$ thick) that electrons pass through them. In spite of these thin walls, these capsules have extraordinary mechanical strength³⁰.

Catalase catalyses the decomposition of H_2O_2 to O_2 and H_2O ³¹. Immersion of a catalase-loaded microcapsule array into a solution containing H_2O_2 causes an immediate decrease in the H_2O_2 concentration (Fig. 3). When the microcapsule array is removed, H_2O_2 decomposition ceases. That this decomposition

is enzyme-mediated is proved by the fact that immersion of a catalase-free capsule array results in no change in the H_2O_2 concentration. Finally, these studies show that the enzyme is not leached from the capsules because, if this were the case, the H_2O_2 concentration would continue to decay after the capsule array was removed from the solution. The issue of enzyme retention will be discussed in greater detail below.

Analogous experiments were done with glucose oxidase (Fig. 4A)³². Curves 'a' and 'b' in Fig. 4A compare catalytic activities for microcapsule arrays containing two different loading levels of glucose oxidase. As would be expected, the capsules with the higher glucose oxidase content show higher enzymatic activity. The ability to control the amount of enzyme immobilized is an important feature of this microcapsule immobilization method. An assay³² of the quantity of glucose oxidase loaded into the capsules used for curve 'a' showed that an amount equivalent to 625 mg glucose oxidase per ml of capsule volume was present in each capsule. It can be shown from the specific volume of glucose oxidase³³ that this quantity occupies 47% of the available volume within a microcapsule. These data show that high concentrations of enzyme can be loaded into these microcapsules.

Enzyme immobilization is a critical issue in the development of new biosensors. A number of proposed glucose biosensors have been prepared by physically entrapping glucose oxidase within polypyrrole films^{11, 20}. Curves 'c' and 'd' in Fig. 4A show the enzymatic activities for two such polypyrrole films¹². The

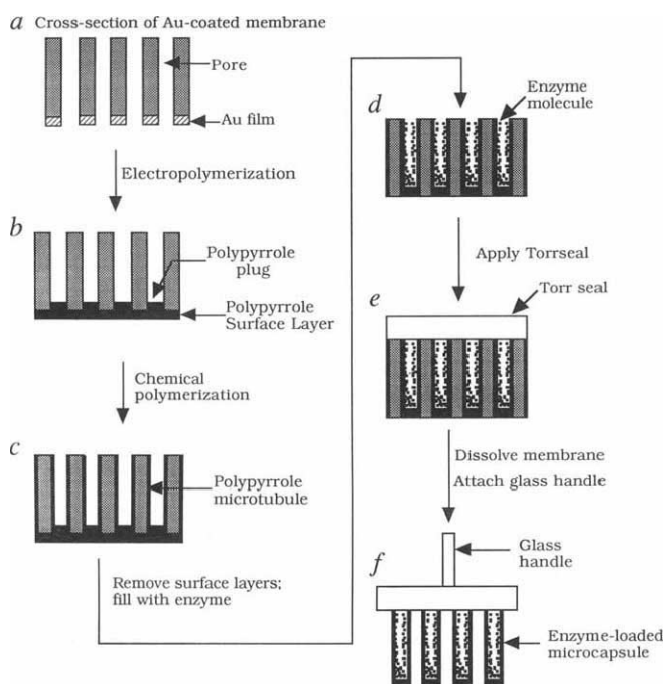


FIG. 1 Schematic diagram of methods used to synthesize and enzyme-load the microcapsule arrays. a, Au-coated template membrane. b, electropolymerization of polypyrrole film. c, Chemical polymerization of polypyrrole tubules. This is accomplished by immersing the membrane into a solution that contains pyrrole monomer and an oxidizing agent (Fe^{3+}). d, Loading with enzyme. e, Capping with epoxy. The epoxy is too viscous to flood the microcapsules. f, Dissolution of the template membrane.

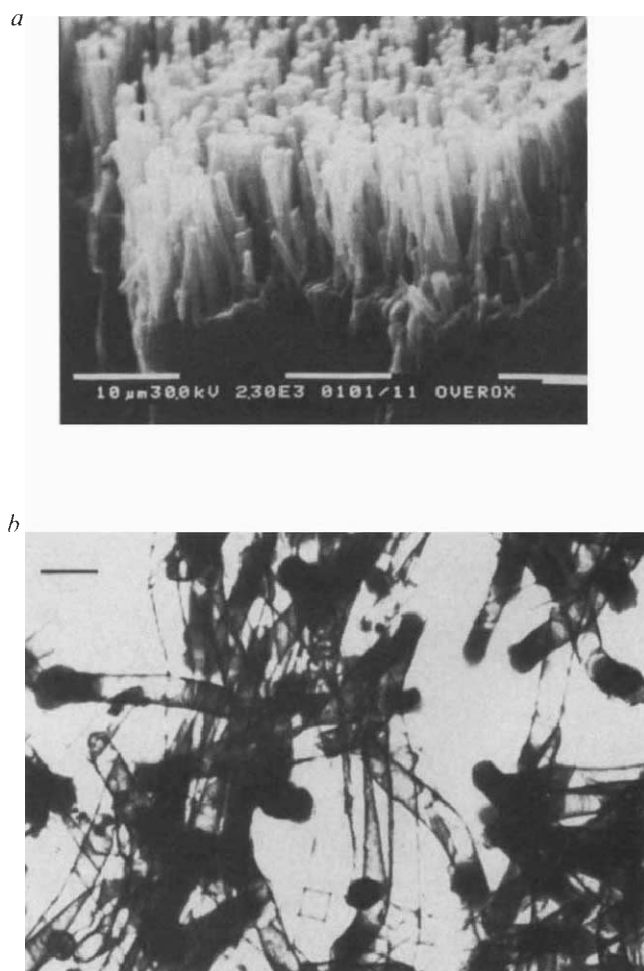


FIG. 2 a, Scanning electron micrograph of a typical microcapsule array. Scale bars, 10 μm . b, Transmission electron micrograph of microcapsules that had not been attached to the epoxy surface. Scale bar in upper left corner, 1.0 μm .

films were $\sim 4.7 \mu\text{m}$ (c) and $0.8 \mu\text{m}$ (d) thick¹⁴. (The activities for these films are nearly the same because only a thin layer ($\sim 0.3 \mu\text{m}$ thick) at the outer surface of the polypyrrole film is enzymatically active^{13,15}.) A comparison of the slopes of curves 'c' and 'd' with the slope of curve 'a' clearly shows that higher enzymatic activity can be achieved with our microcapsule-immobilization method. Hence, our microcapsule arrays show promise for the development of new types of enzymatic biosensors.

There is considerable current interest in the idea of using enzymes in organic solvents³⁴. Because polypyrrole is insoluble in all solvents, these microcapsule arrays should be compatible with any desired solvent. To demonstrate this, we immersed an array of subtilisin-loaded capsules in dry acetone containing *N*-acetyl-L-phenylalanine ethyl ester and propanol; subtilisin should catalyse the transesterification reaction. Transesterification is observed when subtilisin-loaded microcapsules are used and is not observed when the microcapsules are empty (Fig. 4B).

Two important questions remain to be addressed: (1) what is the smallest enzyme that can be immobilized, and (2) how large a substrate molecule will be able to permeate the capsule walls? The enzyme trypsin was used to address the first question. With a molecular mass (M_r) of 23,500 (23.5K) trypsin, which catalyses ester hydrolysis, is one of the smallest of enzymes. The catalytic activity of a freshly prepared array of trypsin-loaded microcapsules was evaluated using *N* α -benzoyl-L-arginine ethyl ester as the substrate molecule. The trypsin-loaded capsule array was then stored in aqueous buffer solution for 45 days at 4°C . The catalytic activity was then re-evaluated; within experimental error, no loss of enzymatic activity was observed. These data show that trypsin is not leached from these capsules. Again, as trypsin is one of the smallest of enzymes, this means that nearly any enzyme can be permanently immobilized within these microcapsules.

The question of size of the permeant molecule was explored using alcohol dehydrogenase. This enzyme catalyses the oxidation of ethanol using nicotinamide-adenine dinucleotide (NAD) as the electron/proton acceptor. Alcohol dehydrogenase was loaded into the microcapsules, and NAD and ethanol were present in the external solution phase. The enzymatic reaction was followed by spectroscopically assaying the external solution for NADH. Experiments analogous to those described in Figs 3 and 4 showed that the dehydrogenase loaded capsules catalyse

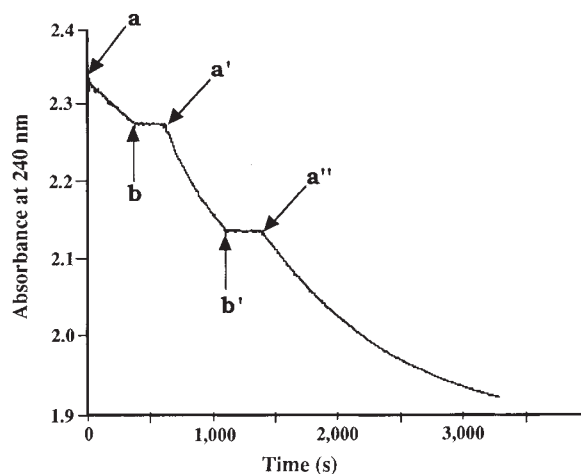


FIG. 3 Evaluation of the enzymatic activity of catalase-loaded capsules. The course of this reaction was followed by monitoring the H_2O_2 absorbance at 240 nm (ref. 31). The curve shows absorbance due to H_2O_2 upon insertion (points a, a', a'') and removal (points b and b') of a catalase-loaded microcapsule array into a solution that was 35 mM in H_2O_2 , pH 7.0 phosphate buffer.

alcohol oxidation. For this to occur, both NAD and NADH must permeate the walls of the microcapsules, and these are large molecules (M_r 660). Because substrate molecules for most enzymatic processes are smaller than this, these experiments show, again, that these tubules can be used with nearly any enzymatic system. Large molecules are able to permeate the capsule wall because polypyrrole is nanoporous³⁵ and because the walls are extraordinarily thin. Finally, to prove that NAD does indeed permeate the capsule walls, we ran analogous experiments with dextran (M_r 40K)-linked NAD in the external solution. No enzymatic activity was observed.

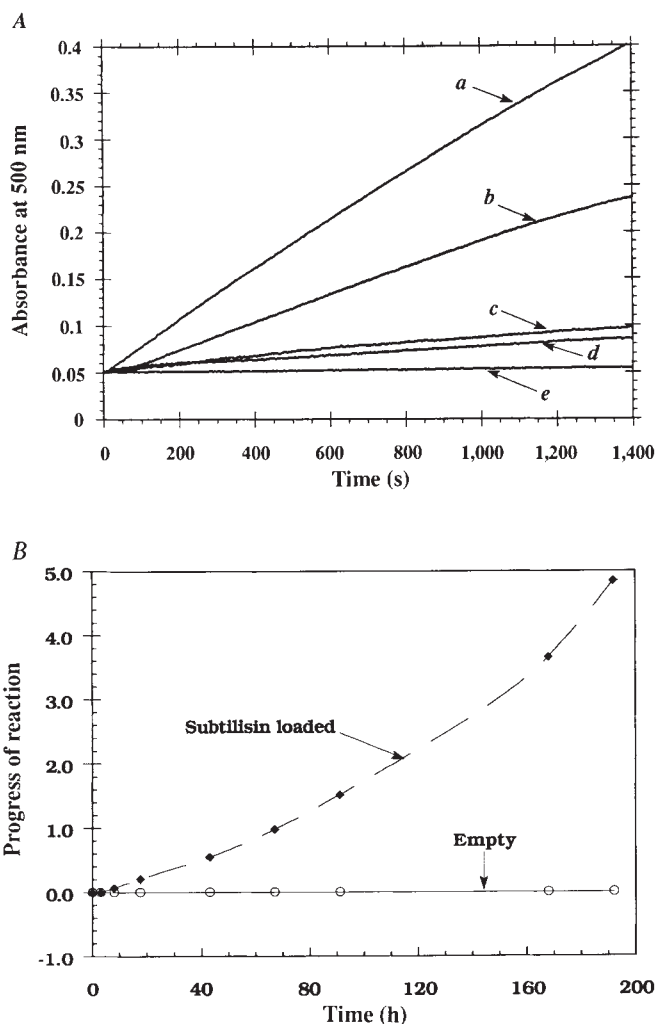


FIG. 4 A, Evaluation of the enzymatic activity of glucose oxidase loaded microcapsules (curves 'a', 'b'), and empty microcapsules (curve 'e'). The standard *o*-dianisidine/peroxidase assay was used³². A larger amount of the oxidase was loaded into the capsules used for curve 'a' than in the capsules used for curve 'b'. Curves 'c' and 'd' are for a competing immobilization method—entrapment within a polypyrrole film^{11–20}. All activities have been normalized so that the geometric areas of the polypyrrole films and the geometric area of the membrane used to prepare the array of microcapsules are the same. All data were obtained after immersion of the oxidase containing system (that is, microcapsule arrays or polypyrrole films) in buffer solution for 24 h at 4°C . This was done to remove any oxidase that is not permanently immobilized. This is a particular problem for glucose oxidase immobilized in the polypyrrole films^{11,17}. B, Evaluation of the enzymatic activity for subtilisin-loaded and empty microcapsules. The progress of the transesterification reaction was followed using gas chromatography³⁴. The solution was 5 mM in ester and 1 M in propanol. The solvent was dry acetone. ('Progress of reaction' on the y-axis is the ratio of the area under the product peak to the area under the reactant peak.)

We have also prepared polymeric microcapsules of length 20 μm , and have tested their catalytic activity when loaded with glucose oxidase. These structures show a higher catalytic activity than the 10- μm capsules, showing that all (or most) of the encapsulated enzyme can be accessed. This contrasts with the case of thin-film immobilization, in which only a surface layer of enzyme is accessible so that the activity is independent of thickness. Thus the activity of our structures can be enhanced simply by increasing the microcapsule length.

We have shown that microcapsule arrays can be prepared via the template synthesis method and that these arrays provide a general approach for enzyme immobilization. This new enzyme immobilization concept should find applications in biosensors and bioreactors. It is worth mentioning that the template method has been used to prepare tubular microstructures composed of a variety of polymers, metals and other materials^{22,23}. Hence it should be possible to prepare bioreactors (of the type described here) that are composed of a variety of different materials. $\square$

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Artificial transmembrane ion channels from self-assembling peptide nanotubes

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NATURALLY occurring membrane channels and pores are formed from a large family of diverse proteins, peptides and organic secondary metabolites whose vital biological functions include control of ion flow, signal transduction, molecular transport and production of cellular toxins. But despite the availability of a large amount of biochemical information about these molecules¹, the design and synthesis of artificial systems that can mimic the biological function of natural compounds remains a formidable task^{2–12}. Here we present a simple strategy for the design of artificial membrane ion channels based on a self-assembled cylindrical β -sheet peptide architecture¹³. Our systems—essentially stacks of peptide rings—display good channel-mediated ion-transport activity with rates exceeding 10^7 ions s^{-1} , rivalling the performance of many naturally occurring counterparts. Such molecular assemblies should find use in the design of novel cytotoxic agents, membrane transport vehicles and drug-delivery systems.

Recently, we described the design and synthesis of a new class of tubular materials based on self-assembling ring-shaped peptide structures¹³. The approach has two inherent advantages: the internal diameter and the outside surface properties of the nanotubes can be controlled simply by adjusting the ring size and the amino-acid composition of the peptide subunit employed¹⁴. Specifically, the ability to tailor the surface characteristics of the tubular ensemble was expected to extend its utility to a variety of biological and materials science applications in which the

physical properties of the media are of paramount importance. One such situation is encountered in the present design of a transmembrane channel structure where the appropriate hydrophobic surface characteristic is essential for stable integration and self-assembly in the nonpolar environment of lipid bilayers¹⁵ (Fig. 1).

According to the design principles previously described¹³, cyclic peptide structures made up of an even number of alternating D- and L-amino acid residues can adopt a flat-ring conformation and stack, under favourable conditions, to furnish a contiguous hydrogen-bonded hollow tubular structure. An ensemble of eight to ten such subunits, each separated by the expected inter-subunit distance of 4.7–5.0 Å and decorated with appropriate hydrophobic surface residues, would be long enough to span the thickness of average biological lipid membranes (Fig. 1). The eight residue cyclic peptide *cyclo*[-(Trp-D-Leu)₃Gln-D-Leu-] was designed for this purpose. It is composed of alternating L-tryptophan and D-leucine side-chain moieties, with the exception of one L-glutamine residue that was introduced mainly to simplify the peptide synthesis¹⁶. It was hypothesized that channel structures, having an aqueous pore of ~7.5 Å diameter, would form spontaneously on incorporating a sufficient concentration of the peptide monomer in lipid bilayers. The driving force for the self-assembly of the channel structure is provided primarily by the enthalpic contribution of a large number of hydrogen-bonding interactions (which are favoured in the low-dielectric-constant medium of lipid bilayers), and also by the increase in the lipid chain entropy arising from side-chain-lipid interactions^{15,17,18}. In short, we rationalized that the designed flat ring-shaped cyclic peptide is not only structurally predisposed toward intermolecular interaction, but is also energetically favoured to self-assemble in the lipid bilayer environment to furnish the desired transmembrane channel structure. The following studies, using a variety of spectroscopic techniques, lipid vesicle model systems and single ion-channel recordings, support the validity of the above design hypothesis.

Addition of the peptide subunit to aqueous liposomal suspensions effects a rapid partitioning of the subunit into the lipid

bilayers, and also its spontaneous self-assembly into ion transport-competent membrane channel structures. Incorporation of the peptide subunit into lipid bilayers using large unilamellar vesicles has been established by absorption and fluorescence spectroscopy. Formation of the putative hydrogen-bonded transmembrane channel structure in phosphatidylcholin liposomes has been supported by Fourier transform-infrared spectroscopy (Fig. 2). The observed amide-I band at $1,624\text{ cm}^{-1}$ is not only at a similar wavenumber to the carbonyl stretching bands found in previously characterized nanotube structures^{13,14} but is also consistent with the infrared spectrum of gramicidin A in similar lipid bilayers¹⁹. Furthermore, the observed N-H stretching band at $3,272\text{ cm}^{-1}$ strongly supports the formation of a tight network of hydrogen bonds with an average inter-subunit distance of $4.7\text{ \AA}$.

Formation of transmembrane channels was also inferred from its highly efficient proton transport activity. Vesicles were prepared having pH 6.5 inside and pH 5.5 in the outside bulk solution. The collapse of the imposed pH gradient in these vesicles, upon formation of the putative transmembrane channel structure, was studied by monitoring the fluorescence intensity of an entrapped pH-sensitive dye²⁰. As shown in Fig. 3, addition of

the peptide to such vesicle suspensions causes a rapid collapse of the pH gradient. According to these experiments, the apparent ion transport activity of *cyclo*[-(Trp-D-Leu)₃-Gln-D-Leu-] is similar to, if not higher than, that of gramicidin A and amphotericin B (Fig. 3). Control studies, monitoring the release of 5(6)-carboxyfluorescein dye, indicated that the collapse of the pH gradient was neither due to the rupturing of the liposomes nor due to the small amounts of organic solvents (<2% dimethylsulphoxide (DMSO)) employed in these studies^{21,22}. Furthermore, the control peptide *cyclo*[-(Gln-D-Leu)₄] (which lacks the appropriate surface characteristics for partitioning into the lipid bilayers, but is otherwise quite similar in design to the channel-forming peptide described above) does not display any ion transport activity under similar conditions. The second control peptide *cyclo*[-MeN-D-Ala-Phe]₄, which has the desirable hydrophobic surface characteristics but lacks the propensity for participating in extended hydrogen bonding network, was also designed and tested for ion transport activity. The peptide design incorporates a novel N-methylation strategy on one face of the ring structure which predisposes the subunit toward a dimeric cylindrical structure (M.R.G., K. Kobayashi, J.R.G., R. Chadha and D. E. McRee, manuscript in preparation). Such a dimeric

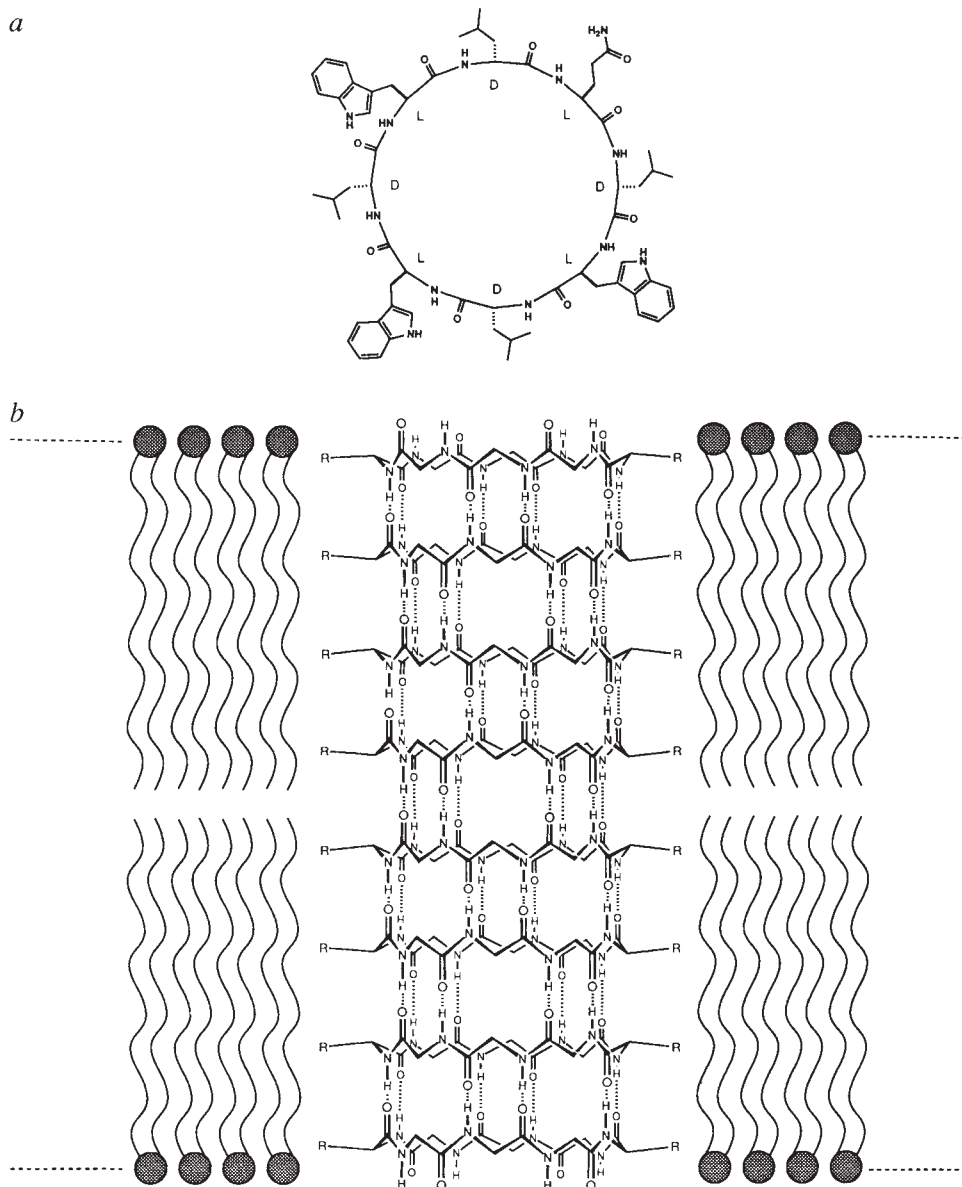


FIG. 1 a, The chemical structure of the peptide subunit is represented in a flat ring-shaped conformation (D or L refers to the amino-acid chirality). b, Peptide subunits are shown in a self-assembled tubular configuration embedded in a lipid bilayer membrane. The representation emphasizes the antiparallel ring stacking, the presence of extensive inter-subunit hydrogen-bonding interactions and side-chain-lipid interactions (for clarity most side chains are omitted).

cylindrical ensemble is ~ 10 Å thick and cannot span the lipid bilayer. Although the peptide has been shown to partition effectively into lipid bilayers, it does not promote proton transport activity in the above vesicle experiments. Together, these experiments suggest that not only the hydrophobic surface character-

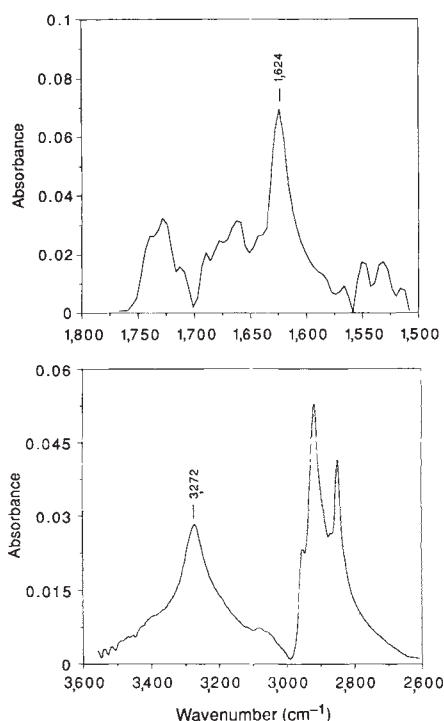


FIG. 2 Infrared spectrum (at 8-cm^{-1} resolution) of **a**, the amide-I region and **b**, the N-H stretch region of the membrane channel structure. Samples were prepared by the addition of a DMSO solution of the peptide to purified liposomes (20–50 phospholipids per peptide subunit) followed by gel filtration on a Sephadex G-25 column (for the method used to prepare the liposomes see Fig. 3 caption). Liposomes were then applied to a CaF_2 disk and dried *in vacuo* for 30 min. Characteristic absorbances due to the peptide channel ensemble are indicated at $1,624$ and $3,272\text{ cm}^{-1}$.

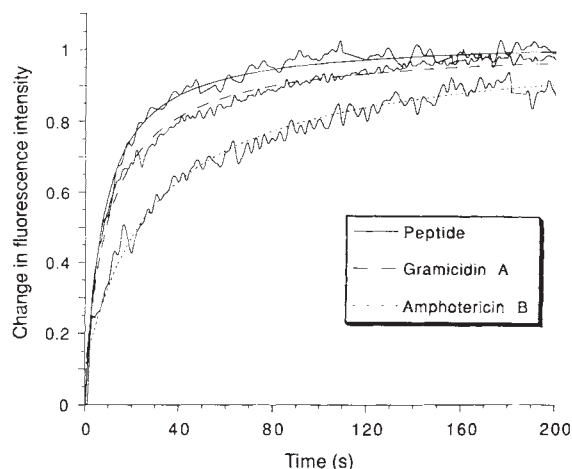
FIG. 3 The ensuing proton efflux on the addition of channel-forming compounds is expressed in terms of the fractional change in the fluorescence intensity of vesicle-entrapped 5(6)-carboxyfluorescein as a function of time (sampled at 0.3-s intervals). Equal molar amounts of channel-forming compounds were used in each experiment to allow direct comparisons of channel-mediated proton transport activity. The amount of added channel-forming compounds in each experiment ranged from 2 to 20 nmol, corresponding to ~ 150 channels per liposome for the lowest concentrations and $\sim 1,100$ channels per liposome for the highest amount of added compounds.

METHODS. Unilamellar vesicles were prepared by the reverse-phase evaporation method²⁷ using 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) and cholesterol in the ratio of 1:1:2 in a solution containing 5(6)-carboxyfluorescein (20 μM in phosphate/saline buffer: 137 mM NaCl, 2.6 mM KCl, 6.4 mM Na_2HPO_4 , 1.4 mM KH_2PO_4 , pH 6.5). Liposomes were then sized by multiple extrusions through polycarbonate membranes²⁸ (10 times, 50 p.s.i., using 0.8 and $2 \times 0.4\text{ }\mu\text{m}$ filter stacks) and the untrapped 5(6)-carboxyfluorescein was removed by size-exclusion chromatography (Sephadex G-25 column $1 \times 30\text{ cm}$) using the same phosphate/saline buffer. Vesicles formed in this way are $\sim 150\text{ nm}$ in diameter as determined by electron microscopy²⁹. In each experiment, 70 μl of the stock vesicle solution ($3.5 \times 10^{-3}\text{ M}$ in phospholipids) was added to pH 5.5 buffer (1.3 ml, 137 mM NaCl, 2.6 mM KCl, 6.4 mM Na_2HPO_4 , 1.4 mM KH_2PO_4) and placed in a 1 cm quartz cuvette inside a thermo-jacketed sample holder of the fluorescence instrument and equilibrated at 25°C for 15 min with gentle stirring.

istic of the channel-forming peptide is an important factor, but also the peptide subunit must be able to participate in extended hydrogen-bonded stacking interactions to produce channel structures long enough to span the lipid bilayer.

The designed transmembrane ensemble also shares important characteristics with natural ion channel formers such as gramicidin A and amphotericin B. First, the peptide shows concentration dependence effects on the rate of channel formation (data not shown). Second, when low concentrations of the channel-forming peptide is used in the above proton transport experiments, only part of the vesicle population goes to equilibrium very fast. This phenomenon reflects the statistical distribution of the channel-forming species among the vesicle population—only part of the population has enough channel-forming molecules to form permeable-competent structures in an all-or-none type of a process. 'Ion carriers' such as monensin and valinomycin bind to metal ions and partition between aqueous and lipid-phases to establish ion equilibrium across the membrane. Unlike these ion carriers, channel-forming species at low concentrations (the designed peptide here, amphotericin B, gramicidin A, and others), because of their inability to diffuse back out of the membrane, cannot penetrate other vesicles; unlike ionophores, they cannot easily establish proton or ion equilibrium in all vesicles present in solution²³. Therefore, the observed rapid proton efflux in the above experiments simply reflects the rate-limiting step of peptide diffusion into the lipid bilayer and self-assembly into ion-transport-competent channel structures, and does not reflect the actual rate of channel-mediated ion transport which can occur on a much faster timescale (see below).

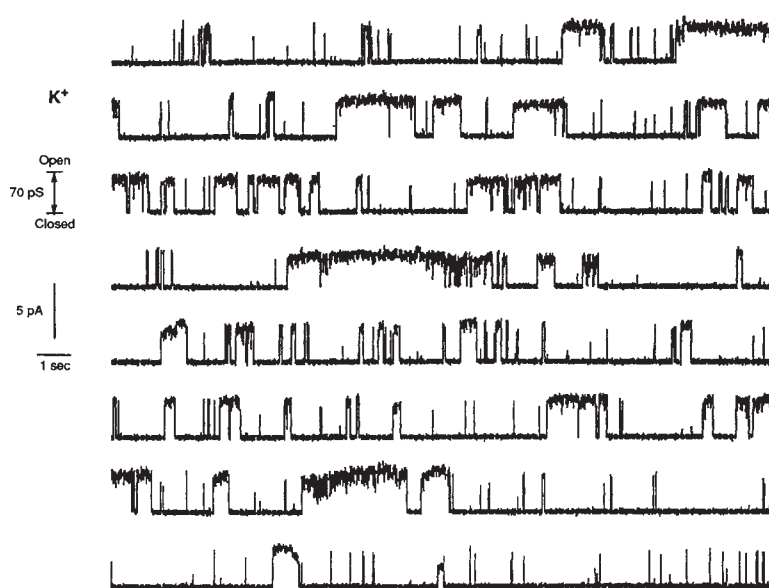
The most critical test for establishing and quantifying the transport efficiency of a membrane channel structure is to measure its single-channel conductance, using micro patch clamp techniques^{1,24}. Observing a high throughput rate of ions demonstrates ion channel formation, and is a diagnostic feature distinguishing ionic channel mechanism from those of other ion transport devices such as ion carriers^{1,25}. Single-channel conductances, using planar lipid bilayers with peptide concentrations of the order of 10^{-7} M in the subphase, are ~ 55 pS (pico Siemens) in 500 mM NaCl, and 65 pS in 500 mM KCl (Fig. 4). The higher conductance found in KCl as compared to NaCl is consistent with the expected weak ion selectivity of the $7.5\text{ }\text{\AA}$ pore structures, and reflects the relative mobility of Na^+ compared to K^+ .



To the cuvette, through an injector port, 25 μl of the channel-forming compounds in DMSO was added with continuous fluorescence monitoring at 520 nm (excitation at 470 nm). Data were then normalized for comparison into the fractional change in fluorescence²⁰ given by $(I_0 - I_t)/(I_0 - I_\infty)$ where I_0 is the initial, I_∞ the final, and I_t the observed fluorescence intensity, respectively.

FIG. 4 A 140-s continuous K^+ single-channel conductance recording at 50 mV. Open-closed transitions indicate gating mechanisms which may reflect structural flexibility or rapid assembly-disassembly of the tubular membrane channel structures. Short and long channel lifetimes (τ) in the open state could be distinguished, with $\tau_{\text{short}} = 0.71 \pm 0.03$ ms and $\tau_{\text{long}} = 37.14 \pm 8.99$ ms (total number of events 510) with an overall open probability P_o of 0.29. Peptide concentration in the subphase was 1.0×10^{-7} M. As expected for any such self-assembling channel-forming species, channel opening lifetimes show significant dependence on the peptide concentration. Raising the added peptide concentration in the subphase to 2.0×10^{-5} M produces open channel lifetimes of >30 s.

METHODS. The lipid bilayers used were formed on the tip of patch pipettes using a mixture of synthetic POPE:POPS (4:1) (1-palmitoyl-2-oleoyl-Sn-glycero-3-phosphatidylethanolamine and -serine). Aliquots (5–10 μ l) of the peptide solution (1.0×10^{-7} or 2.0×10^{-6} M in 25% DMSO in buffer solution containing 500 mM NaCl or KCl, 5 mM $CaCl_2$, 10 mM HEPES, pH 7.5) were added to 150 μ l subphase volume resulting in spontaneous partitioning of the peptide into the membrane. Ion channels formed spontaneously after peptide was added to the subphase of lipid bilayers. Ion channel activity was observed in 14 out of 22 membranes under symmetrical solutions of 500 mM NaCl or KCl, 5 mM $CaCl_2$, 10 mM HEPES, pH 7.5. Data acquisition and analysis were performed on a Gateway2000/486 computer using the pClamp



software package and TL-1 Labmaster interface. Data were acquired at intervals of 0.1 ms and filtered at 2 kHz.

ions in solution. Therefore a 55-pS channel in NaCl and a 65-pS channel in KCl are likely to arise from the same structural entity (same number of stacked rings). In addition, the single-channel conductance was shown to be independent of the applied voltage in the measured range of 10–100 mV. The actual rate of channel-mediated ion transport is therefore a prodigious 2.2×10^7 ions s^{-1} for K^+ and 1.8×10^7 ions s^{-1} for Na^+ , which is almost three times faster than that of gramicidin A under similar conditions²⁶.

The strategy described here should allow for the design of transmembrane channel structures with larger pore diameters for use in 'molecular' transport across lipid bilayers; as such it should provide a potential vehicle for drug delivery into living cells and may find use in antisense and gene therapy applications. □

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Biochemical indicators of diagenetic alteration in natural organic matter mixtures

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THE extent of degradation of natural organic mixtures largely determines their utility as records of depositional history, and their potential to act as nutritional substrates and fossil fuel sources. The degree of decomposition is usually inferred from the physical setting of the deposits¹ or from bulk chemical composition², but in both these cases the interpretation can be obscured by several factors³. We report here a study of aldoses and amino acids in samples collected from a variety of marine depositional environments and representing widely different stages of alteration. We identify consistent trends in three compositional characteristics: the percentage of organic carbon in the form of aldoses and protein

amino acids, the percentage of total nitrogen present as protein amino acids, and the percentage of total amino acids present as β -alanine and γ -aminobutyric acid. Each of these parameters is sensitive to a different stage of alteration, and they appear to be uncompromised by source variations. Applied together, they offer concordant information on the relative diagenetic stage and reaction potential of natural organic mixtures under both aerobic and anaerobic depositional conditions.

Over 99% of global primary organic matter production is ultimately respired to CO_2 and inorganic nutrients^{4,5}, a process that ceases only when a small residue is preserved, primarily in marine sediments⁵. Most organic materials in plant litter, soils, natural waters and surficial sediments therefore are transients at some intermediate stage of degradation. These materials are also heterogeneous, often containing recently introduced, fresh material as well as older, more refractory components.

Information on the alteration history of natural organic mixtures is important from both biological and geochemical perspectives. It may be useful in identifying their nutritional potential and the nature of past alteration processes, and in deciphering sedimentary organic records and factors controlling fossil fuel formation. Diagenetic indicators might also help to discern whether steady-state deposition has been maintained at a given site and whether down-core trends in organic content result from changes of input or preservation, or from varying physical processes (for example, mineral dilution or winnowing). Site comparisons are particularly challenging because sources may differ, absolute ages may not be tracked by diagenetic maturity, and superposition may not be applicable. Other variables, such as redox conditions or bioturbation, may also confound comparisons.

To date, a number of indicators have been used as relative estimates of organic reactivity. These include the physical setting, such as position relative to a source or within a core, but interpretation may be confused by physical processes such as mixing, differential transport or variable deposition. Bulk chemical properties, such as C/N ratios or contents of hydrolysable or humic materials, have also been used, but also have liabilities. Changes in C/N ratios can be caused not only by commonly observed preferential loss of nitrogen but also by source changes (for example, marine or terrestrial) and inorganic nitrogen immobilization⁶. Hydrolysable or humic material contents are operationally defined, chemically ambiguous and do not necessarily reflect bioavailability or reactivity. Organic carbon contents have also been used to compare diagenetic maturity, but again may be complicated by physical processes, and bulk elemental analyses offer little or no mechanistic information.

Because the precursors of decaying organic material are biopolymers that differ in composition, solubility and resistance to microbial attack⁷, advancing stages of degradation may be characterized by trends in biochemical composition. Selected lipids⁷, pigments⁸ and lignins⁹ undergo either preferential loss (for example, chlorophyll) or preservation (for example, lignin) in patterns that have found use as maturity indicators in selected soils and recent sediments¹⁰⁻¹². However, these diagenetic trends are often process-specific and most of the individual molecules involved have limited distributions and/or are trace components. Many of these biochemical trends are therefore diagnostic only in specific situations and may not reflect the average history of natural samples that typically have multiple sources. Although a number of primarily lipid-based biochemical maturity indicators have been developed for kerogens and fossil fuels¹³, no established, generally applicable indicators are currently available for organic mixtures undergoing early diagenetic alteration.

Proteins and polysaccharides offer the potential advantage that they are important organic components of both terrestrial and marine organisms as well as environmental mixtures^{14,15}. A recently developed method for assessing the content of intact protein in natural samples¹⁶ (thereby providing a direct measure

of substrate quality) offers great promise, but is labour intensive and complicated by interference from humic substances. Methods for amino acids and aldoses do not have these disadvantages and have revealed a number of apparently degradation-related compositional trends. The percentage of total organic carbon (TOC) represented by hydrolysable amino acids (AA) or aldoses (SUG) (here combined as $\%(T_{AA} + T_{SUG})OC$) and the percentage of total nitrogen present as the same amino acids ($\%T_{AA}N$) have been reported to drop as diagenesis progresses (for example, refs 17-22). In contrast, mole percentages of the non-protein amino acids β -alanine (BALA) and γ -aminobutyric acid (GABA) (here combined as $\%(BALA + GABA)$) typically increase relative to their more abundant protein counterparts (refs 18-25). Previous studies, however, were typically restricted to specific sample types and redox conditions, and to limited diagenetic sequences. Moreover, interlaboratory analytical differences often make published amino acid and aldose yields and compositions difficult to compare, and natural samples have rarely been directly compared to potential source organisms. The observed compositional trends and their causes therefore have yet to be confirmed by systematic comparisons, using uniform analytical methods, of natural samples from different depositional systems that cover a wide range of diagenetic states. Consequently, the general applicability of these parameters and the extent to which they may be compromised by source changes remain untested, and none have found routine application, alone or combined.

We have examined the above three compositional parameters across a broad range of diagenetic conditions (Fig. 1). These extend from unaltered marine and terrigenous organisms to slow oxidation in abyssal turbidites from the eastern Atlantic. In the latter case, oxygen has diffused into previously reducing sediments over thousands of years²⁶ creating 'oxidation fronts' in initially homogeneous sediments. Our sample set includes suites of materials from two coastal sites, Saanich inlet (British Columbia, Canada) and Dabob bay (Washington, USA), where bottom waters are respectively anoxic and oxygenated, and overlay varved compared to bioturbated sediments^{14,27}.

The samples are presented in a diagenetic sequence suggested by the nature of the material or its physical setting. Samples from Saanich inlet and Dabob bay include water-column particles (annual-average sediment trap materials) and surficial and deep sediments (top and bottom intervals from gravity- and box-cores, respectively). These sites have similar plankton populations and rates of primary production and sedimentation, and both receive considerable terrigenous inputs^{14,15,25,27}. Combined with the suite of source organisms, these samples provide a well-constrained test of the proposed indices over a wide range of early diagenetic alteration, with mixed sources and differing redox conditions. Although its sources may differ, organic matter in the Atlantic turbidite samples is also originally derived from a productive coastal zone²⁶. This sample set therefore provides a meaningful extension of the Saanich inlet and Dabob bay diagenetic sequences; it also allows the effects of slow aerobic degradation to be isolated in a rare case where steady-state deposition need not be assumed. Ranges and averages for the source organism suite are also presented (Fig. 1). Average values for diatoms, major contributors to phytoplankton populations and water-column particle fluxes in both Saanich inlet and Dabob bay, are included as points of reference for these sites. Organic carbon contents ($\%OC$) range over two orders of magnitude (Fig. 1) from the intact organisms (22-49%) to the reducing ($\sim 1\%$) and oxidized turbidite samples ($\sim 0.2\%$), and display a gradual decrease consistent with the presumed diagenetic sequence.

All three parameters give consistent and complementary indications of diagenetic history (Fig. 1). The progressive decrease in $\%OC$ is tracked by $\%(T_{AA} + T_{SUG})OC$ and $\%T_{AA}N$, and mirrored by $\%(BALA + GABA)$. With only one exception ($\%T_{AA}N$ for trap materials), all values for the Saanich and Dabob samples

are concordant with %OC differences for the three sample pairs, despite contrasting redox conditions. Values for all parameters ultimately fall well outside ranges observed for intact organisms (notably, only woody vascular plant tissues have %T_{AA}N values below 48%), thus providing uncompromised indications of the extent of alteration, even in its earlier stages (Fig. 1).

These indices are quite distinct in their diagenetic sensitivities. By incorporating both amino acid and aldose yields, % (T_{AA} + T_{SUG})OC reflects behaviour of the major fraction of potentially reactive biochemicals in all samples, making it sensitive to the presence of relatively unreactive materials such as lignin, humin or fossil organic matter. Aldose or amino acid carbon contributions have previously been used individually as reactivity indicators in riverine and marine samples^{21,28}. Combining their yields produces a parameter that reflects a larger portion of the total organic material, but clearly decreases during alteration of both marine and terrigenous organic materials. This removes possible interpretive ambiguities for samples with mixed inputs caused, for example, by amino acid yields that can rise in degrading terrigenous organic materials and soils (G.L.C. and J.I.H., unpublished data and ref. 29). Changes in aldose and amino acid compositions also occur and indicate selective preservation of diatom cell-wall polymers across the benthic interfaces of both Dabob bay and Saanich inlet^{15,25}, but they are otherwise variable and not definitive.

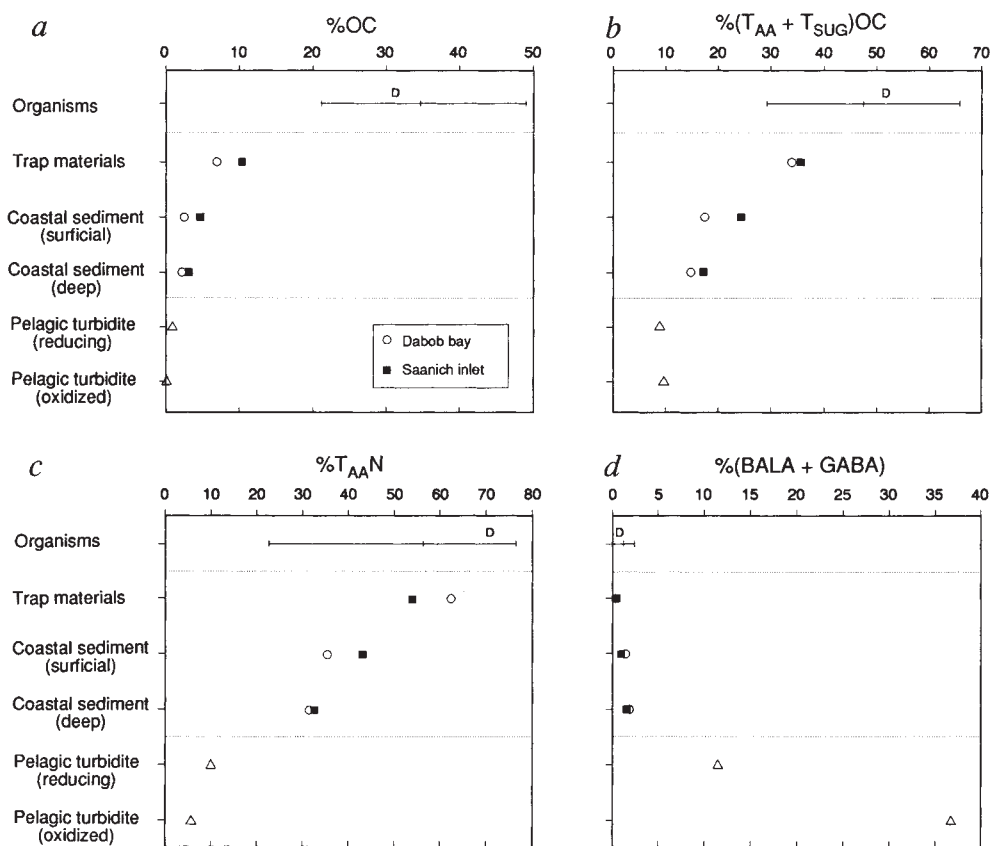
By normalizing to total nitrogen, %T_{AA}N values are diagnostic of an important nutrient element, and are likely to be low in heavily altered samples where contributions of diagenetically immobilized nitrogen can be important⁶. This parameter there-

fore bypasses the potential ambiguity of C/N ratios. Diagenetic partial decarboxylation of aspartic and glutamic acids^{22,24} has previously been suggested as a source of β -alanine and γ -aminobutyric acid, respectively; inverse concentration trends often observed for the parent-daughter pairs (for example, Fig. 2) are consistent with this postulated source. Few persistent organic by-products of diagenesis have been definitively identified to date, and % (BALA + GABA) may therefore prove to be an unusually sensitive and unambiguous indicator of alteration. The respective daughter-to-parent ratios (for example, β -alanine/aspartic acid) displays trends similar to that of % (BALA + GABA) in this sample set. Caution should be taken in using such ratios as diagenetic indices, however, because the aspartic- and glutamic-acid contents of organisms are variable¹⁵ and because, in many cases, partial decarboxylation probably is not their principal fate.

The parameters also appear to be sensitive at different stages of alteration. Changes in % (T_{AA} + T_{SUG})OC values are smaller in the later stages, whereas % (T_{AA}N values decrease steadily over the whole %OC range. In the case of % (BALA + GABA), values differ only slightly in modern materials but increase dramatically in the ancient turbidite samples, to the extent that yields of these two non-protein amino acids ultimately exceed those of all protein amino acids.

Results of previous studies often are not directly comparable due to differences in analytical methods, but trends and absolute values for all three parameters are typically very consistent with the results of this study (refs 17–22,24,30–32). For example, aldose and amino-acid carbon and nitrogen contributions to

FIG. 1 Organic-matter compositional characteristics of a suite of natural samples of differing diagenetic maturity. *a*, Weight per cent organic carbon (%OC), *b*, percentage of organic carbon in the form of hydrolysable primary protein amino acids and aldoses, % (T_{AA} + T_{SUG})OC, *c*, percentage of total nitrogen in the form of hydrolysable primary protein amino acids, %T_{AA}N, *d*, mole percentage of total hydrolysable amino acid yield in the form of the non-protein amino acids β -alanine and γ -aminobutyric acid, % (BALA + GABA). The upper portion of each plot includes a range of values for a suite of fresh organisms (including cultured phytoplankton, bacteria and white-rot fungi, net-tow zooplankton, marine macrophytes, and woody and non-woody angiosperm and gymnosperm tissues^{14,15}, and average values for the entire sample suite and for cultured diatoms (D). Below this are plotted values for three samples each from two coastal sites, Dabob bay (Washington, USA) and Saanich inlet (British Columbia, Canada), which have oxygenated and anaerobic bottom waters respectively. The samples include sediment trap materials (annual averages from year-long, monthly-interval deployments at 30 and 50 m, respectively) and surficial (0–1 cm) and deep (48–50 cm in Dabob bay, 78–80 cm in Saanich inlet) sediment intervals from box (Dabob) or gravity (Saanich) cores. These deep intervals represent ages of ~100 and 120 years before present, respectively. See refs 14, 27 and 35 for site, sample and method descriptions. The lowest part of each plot presents values for samples from either side of 'oxidation fronts' observed in turbidites from the Madeira abyssal plain. The values



(shown as hollow triangles) represent averages of single samples on the oxidized side and pairs of samples on the reducing side of 'f-turbidite' oxidation fronts from two different cores (86P5 and 86P25, see ref. 26 for site, core and method descriptions).

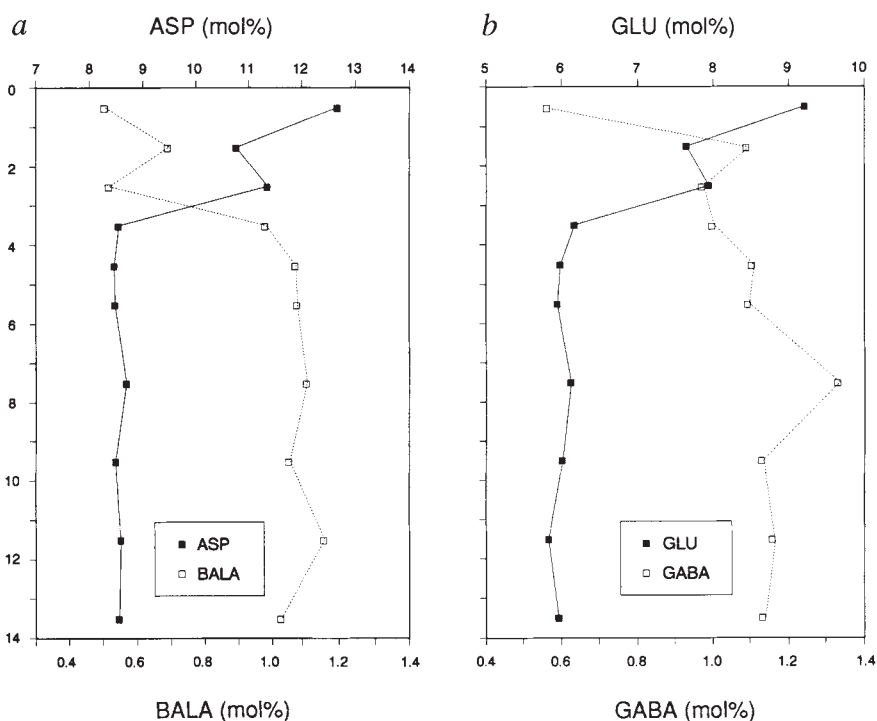


FIG. 2 Mole percentages of *a*, aspartic acid (ASP) and β -alanine (BALA) and *b*, glutamic acid (GLU) and γ -aminobutyric acid (GABA), in the uniformly-varved upper 14 cm horizon of Saanich inlet sediments^{25,27}.

sediment-trap materials from the Sargasso sea²¹ and Panama basin³¹ vary both with season and depth, and are similar to values for coastal trap and surficial sediment samples found in this study (Fig. 1). Absolute values and down-core decreases previously observed in sedimentary %T_{AA}N and in aldose and amino acid carbon contributions also are comparable to results presented here (for example, refs 17–22,30,32), the decreases depending on the extent to which diagenesis is masked by bioturbation^{15,18,19,25}. Down-core increases in %(BALA+GABA) are common and have been observed to reach values as high as 71% in highly degraded pelagic clays²².

The results presented here confirm that the three highlighted parameters are diagenetically sensitive and provide concordant information that is uncompromised by source or redox changes. But due to differences in their nature and in the diagenetic stage at which they are most sensitive, the parameters are most valuable when applied together. Collectively they reflect multiple facets of the degrading material and yield more reliable indices

of alteration, as well as means for independently verifying inferences drawn from any of the individual parameters. Applied jointly, and possibly combined with other indicators such as lignin phenol acid-to-aldehyde ratios^{9,33} or the extent of D–L amino acid racemization³⁴, they should provide complementary indications of the diagenetic maturity of a wide variety of organic mixtures.

Such internally-normalized molecular parameters avoid the limitations of previously discussed physical and bulk chemical indicators of diagenetic maturity. Comparisons among sites, when %OC, C/N ratio, position and age comparisons may be problematic, should be particularly facilitated, as should estimating the extent to which biomarker information may be compromised by diagenesis. Finally, these parameters should help to delineate the relative effects of diagenesis in determining organic matter distribution in soils and sediments, thereby complementing studies of bioactive element cycling in modern and ancient environments. □

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A conservative tracer for glacial ocean circulation from carbon isotope and palaeo-nutrient measurements in benthic foraminifera

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THE ratio of cadmium to calcium (Cd/Ca) and the carbon isotope ratio ($\delta^{13}\text{C}$) in the calcite tests of benthic foraminifera both record nutrient distributions in the ocean^{1,2}. Strict interpretation of both $\delta^{13}\text{C}$ and Cd as nutrient tracers has led to conflicting views of glacial ocean circulation³⁻⁵. Here we show that, when one takes into account the fact that $\delta^{13}\text{C}$ reflects air-sea exchange as well as nutrient distributions, these two proxies can provide complementary information about the movement of deep water in the glacial ocean. We use the Cd concentration (assumed to be controlled primarily by biological cycling) to infer the age history of glacial deep water, and deduce the sources of deep water from the carbon isotope air-sea exchange signature, a conservative tracer that we construct using both Cd and $\delta^{13}\text{C}$ measurements. Our analysis suggests that there were at least two sources of glacial deep water: a less dense component originating in the North Atlantic Ocean, and a more dense component which may have originated in the Pacific Ocean. As well as demonstrating the potential of this approach, our findings provide further support for a Pacific glacial deep water source, evidence for which has until now been both scarce and conflicting^{3,5-12}.

The distribution of cadmium in the ocean resembles that of the major nutrient, phosphorus. Although the mechanism by which Cd becomes incorporated into organisms is not understood, the relationship between Cd and P in the deep ocean is very strong, although not quite linear¹. Foraminifera incorporate varying amounts of Cd into their calcite tests, depending primarily on seawater Cd concentrations and the depth of calcification^{1,5}. Core-top calibrations show that foraminiferal Cd/Ca ratios can be used to estimate seawater Cd to an accuracy of $\sim \pm 0.1 \text{ nmol kg}^{-1}$ (ref. 5).

The isotope composition of carbon in sea water also follows the nutrient distribution within the ocean, with low $\delta^{13}\text{C}$ values corresponding to nutrient-rich waters and high $\delta^{13}\text{C}$ to nutrient-depleted waters². Photosynthesis discriminates against the heavier carbon isotope, and as this isotopically light organic matter oxidizes in the deep sea, the deep water gains nutrients and the $\delta^{13}\text{C}$ of dissolved inorganic carbon decreases. However, seawater $\delta^{13}\text{C}$ can also be altered by exchange with atmospheric CO_2 without associated changes in nutrient concentration. Isotope equilibrium of surface ocean waters with atmospheric CO_2 will cause higher $\delta^{13}\text{C}$ in cold surface waters, and lower $\delta^{13}\text{C}$ in warm surface waters^{13,14}. Deep-water $\delta^{13}\text{C}$ is recorded by benthic foraminifera as oceanic carbon is incorporated into their calcite tests. *Planulina wuellerstorfi* has been shown to record seawater $\delta^{13}\text{C}$ to an accuracy of $\sim \pm 0.2\text{‰}$ (ref. 15).

The air-sea exchange signature, $\delta^{13}\text{C}_{\text{as}}$, can be isolated by subtracting the $\delta^{13}\text{C}$ predicted for an ocean with no air-sea carbon isotope exchange from the actual $\delta^{13}\text{C}$ (ref. 13). The oxidation of organic material within a parcel of water will change both the concentration of phosphate (PO_4^{3-}) and $\delta^{13}\text{C}$, but leave $\delta^{13}\text{C}_{\text{as}}$ unchanged. This means that $\delta^{13}\text{C}_{\text{as}}$ is a conservative tracer in the deep sea, its distribution only reflecting the relative mixtures of water from different sources. ($\delta^{13}\text{C}_{\text{as}}$ is the same as $\Delta\delta^{13}\text{C}$ defined by Broecker and Maier-Reimer¹³, but is renamed here to avoid confusion with the common usage of $\Delta\delta^{13}\text{C}$ to

denote the difference between $\delta^{13}\text{C}$ in surface and deep waters. $\delta^{13}\text{C}_{\text{as}}$, as defined by Broecker and Maier-Reimer¹³, is actually not quite conserved, with small non-conservative behaviour introduced by the assumption of constant dissolved inorganic carbon throughout the ocean, and the assumption of constant $\delta^{13}\text{C}$ of organic material.) Because of the close relationship between the concentrations of Cd and PO_4^{3-} in the ocean, we can calculate $\delta^{13}\text{C}_{\text{as}}$ using $\delta^{13}\text{C}$ and Cd estimates from benthic foraminifera. Here we use the global compilation of $\delta^{13}\text{C}$ and Cd estimates of Boyle⁵ to calculate the distribution of $\delta^{13}\text{C}_{\text{as}}$ in the modern and glacial ocean (Table 1, Fig. 1).

The distribution of $\delta^{13}\text{C}_{\text{as}}$ calculated for the modern ocean (Fig. 1c) is consistent with previous estimates^{13,16}. We can see that there are at least two sources of water to the deep ocean, each with its own distinct $\delta^{13}\text{C}_{\text{as}}$ signature. The North Atlantic source has a low $\delta^{13}\text{C}_{\text{as}}$ signature (-0.4‰), reflecting a lesser influence of equilibration at low temperatures than average deep water. The Southern Ocean source has a higher $\delta^{13}\text{C}_{\text{as}}$ signature ($+0.2\text{‰}$), reflecting the greater influence of equilibration of this water with the atmosphere at low temperatures^{13,14}. The Cd estimates reflect the regeneration of organic material into the deep ocean waters from their ultimate origin in the Atlantic, through the Southern Ocean and into the deep Indian and Pacific Oceans. Although they also reflect the different $\delta^{13}\text{C}_{\text{as}}$ values for the deep-water sources, the $\delta^{13}\text{C}$ estimates also (to first order) reflect this nutrient regeneration pattern.

Estimates of glacial $\delta^{13}\text{C}_{\text{as}}$ require at least two sources of deep water, one with a high $\delta^{13}\text{C}_{\text{as}}$ signature ($\sim +0.8\text{‰}$) and one with a low $\delta^{13}\text{C}_{\text{as}}$ signature ($\sim -0.6\text{‰}$) (Fig. 1f). Intermediate values of $\delta^{13}\text{C}_{\text{as}}$ (eastern tropical Pacific, Indian Ocean, Deep Atlantic probably represent a mixture of waters from these two sources). By definition, the mean glacial deep ocean $\delta^{13}\text{C}_{\text{as}}$ was 0‰ , implying roughly equal contributions from the high and low $\delta^{13}\text{C}_{\text{as}}$ sources. Because of the wide range in $\delta^{13}\text{C}_{\text{as}}$ values for the glacial deep-water sources (1.4‰ in the glacial ocean relative to the modern spread of 0.6‰), $\delta^{13}\text{C}$ no longer reflects the nutrient regeneration implied by the deep-water Cd estimates. However, this wide range in glacial $\delta^{13}\text{C}_{\text{as}}$ values makes this property an excellent conservative water-mass tracer. Understandably, the increased range in $\delta^{13}\text{C}_{\text{as}}$ has confounded attempts to reconstruct deep-water circulation changes using $\delta^{13}\text{C}$ exclusively.

Deep waters displaying $\delta^{13}\text{C}_{\text{as}}$ values near the high ($+0.8\text{‰}$) end member resided in the shallower portions (1–2 km) of the Atlantic and Pacific Oceans. In the Atlantic, this water mass had low Cd values (0.2 nmol kg^{-1}), indicating a likely source region in the North Atlantic. This water mass was probably the glacial North Atlantic intermediate water (GNAIW)³. Although a shallow high- $\delta^{13}\text{C}_{\text{as}}$ water mass also occupied the Pacific Ocean, in the Pacific this water mass had very high Cd values (0.8 nmol kg^{-1}) indicating either a greater distance from the source for this water mass in the Pacific, or higher nutrient values at the time of formation. So it is possible that the GNAIW was not only a dominant water mass in the Atlantic, but made its way into the upper deep waters of the Pacific as well. This communication of low-density deep water between the Atlantic to the Pacific could have been accomplished by way of the lower density (northward) portions of the Antarctic circumpolar current. It is possible that a separate high- $\delta^{13}\text{C}_{\text{as}}$, high-preformed-nutrient water mass occupied the upper glacial Pacific, possibly reflecting the contributions of high $\delta^{13}\text{C}_{\text{as}}$ Southern Ocean surface waters.

Low $\delta^{13}\text{C}_{\text{as}}$ values were concentrated not only in the deep Antarctic, but also in the deep Pacific. Glacial Cd values for these water masses in the Antarctic and North Pacific are quite similar, and therefore cannot be used to distinguish the source. Although the 'anomalously low' glacial $\delta^{13}\text{C}$ is thought of as peculiar to the Southern Ocean, it seems unlikely that this was the source area for the low- $\delta^{13}\text{C}_{\text{as}}$ water. In both the modern and glacial oceans, Antarctic surface water $\delta^{13}\text{C}_{\text{as}}$ is much higher (by $\sim 1\text{‰}$) than that of the deep water which upwells into the

TABLE 1 Oceanic Cd concentrations and carbon isotope ratios

	Depth (km)	Modern Cd (nmol kg ⁻¹)	Modern $\delta^{13}\text{C}$ (‰)	Modern $\delta^{13}\text{C}_{\text{as}}$ (‰)	Glacial Cd (nmol kg ⁻¹)	Glacial $\delta^{13}\text{C}$ (‰)	Glacial $\delta^{13}\text{C}_{\text{as}}$ (‰)
North Atlantic	1–2	0.20	1.10	–0.35	0.20	1.60	0.69
	2–3	0.25	1.00	–0.31	0.23	1.00	0.17
	3–4	0.26	1.00	–0.28	0.37	0.60	0.15
	4–5	0.28	0.90	–0.33			
Tropical Atlantic	1–2	0.25	0.85	–0.46	0.20	1.60	0.69
	2–3	0.30	0.85	–0.32	0.30	0.70	0.06
	3–4	0.32	0.83	–0.29	0.55	0.20	0.24
	4–5	0.35	0.78	–0.26	0.50	0.00	–0.10
Antarctic	surface	0.5	1.6	1.0	0.5	0.8	0.71
	1–2	0.30	0.70	–0.47			
	2–3	0.30	0.70	–0.47	0.40		
	3–4	0.50	0.60	–0.03	0.55	–0.40	–0.36
North Indian	4–5	0.62	0.40	0.10	0.47	–0.40	–0.58
	1–2	0.80	–0.10	0.10	0.55	0.20	0.24
	2–3	0.75	0.00	0.06	0.60	–0.50	–0.32
	3–4	0.70	0.10	0.03	0.50		
Northwest Pacific	4–5	0.70	0.10	0.03			
	1–2	0.84	–0.15	0.16	0.80	0.25	0.97
	2–3	0.82	–0.10	0.16	0.55	–0.10	–0.06
	3–4	0.75	0.05	0.11	0.45	–0.30	–0.53
East tropical Pacific	4–5	0.75	0.05	0.11			
	1–2	0.83	–0.10	0.18	0.87	–0.15	0.77
	2–3	0.75	0.05	0.11	0.65	–0.30	0.01
	3–4	0.70	0.15	0.08	0.65	–0.30	0.01
	4–5	0.70	0.15	0.08			

Subsurface $\delta^{13}\text{C}$ and Cd inventory estimates from Boyle⁵ who used a depth-dependent distribution coefficient to estimate seawater Cd from the foraminiferal Cd/Ca ratio. Values that were extrapolated from adjacent depth intervals were not used. Modern surface Antarctic $\delta^{13}\text{C}$ values are from Kroopnick *et al.*²⁰. Modern surface Cd values were estimated from the global PO_4^{3-} –Cd relationship¹. Glacial surface $\delta^{13}\text{C}$ is calculated by subtracting 0.8‰, the glacial/interglacial $\delta^{13}\text{C}$ difference recorded in planktonic foraminifera¹⁴, from the modern value. Glacial surface Cd is taken to be the same as the modern value, reflecting the lack of glacial/interglacial change in the Cd/Ca ratio recorded in Southern Ocean planktonic foraminifera^{23,24}. Modern $\delta^{13}\text{C}_{\text{as}}$ values are calculated using the equation of Broecker and Maier-Reimer¹³ for $\delta^{13}\text{C}_{\text{as}}$ (their $\Delta\delta^{13}\text{C}$) along with the Cd– PO_4^{3-} relationship for waters containing $>1.3 \mu\text{mol PO}_4^{3-}$ per kg (ref. 1) ($\delta^{13}\text{C}_{\text{as}} = \delta^{13}\text{C} + (2.75 \times \text{Cd}) - 2.0$). For glacial values, the $\delta^{13}\text{C}_{\text{as}}$ equation is modified to account for an oceanic Cd concentration which is 13% lower⁵, organic-matter $\delta^{13}\text{C}$ which 2‰ higher²⁵, a mean ocean $\delta^{13}\text{C}$ which was 0.3‰ lower^{4,5}, and a corresponding increase in total inorganic carbon of 4% ($\delta^{13}\text{C}_{\text{as}} = \delta^{13}\text{C} + (2.73 \times \text{Cd}) - 1.5$). The oceanic phosphate inventory is assumed to be the same as today.

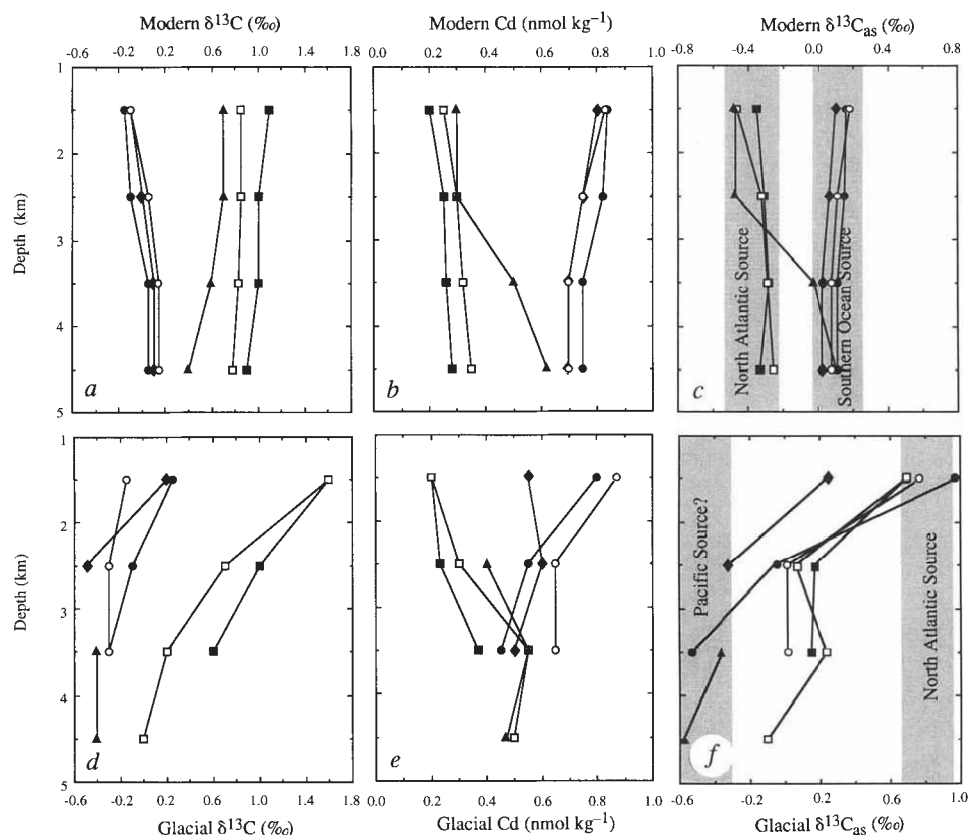


FIG. 1 Values of $\delta^{13}\text{C}$, Cd concentration and estimated $\delta^{13}\text{C}_{\text{as}}$ values for the modern (a–c) and glacial (d–f) oceans (Table 1). Symbols used: Filled squares, North Atlantic; empty squares, tropical Atlantic; filled triangles, Antarctic; filled diamonds, North Indian; filled circles, northwest Pacific; empty circles, eastern tropical Pacific. Differences between Cd concentrations $<0.1 \text{ nmol kg}^{-1}$, and between $\delta^{13}\text{C}_{\text{as}}$ and $\delta^{13}\text{C}$ values $<0.2\text{‰}$, are beyond the limits of the errors of the methods.

surface ocean (Table 1). So surface processes would, if anything, imprint a high $\delta^{13}\text{C}_{\text{as}}$ signature on deep water formed in the region. In the extreme case where deep waters formed exclusively from these relatively well equilibrated surface waters, the new deep waters would have the same $\delta^{13}\text{C}_{\text{as}}$ signature as the surface water (1‰ today, 0.7‰ during the last glaciation). In the extreme case where new deep waters formed in the Southern Ocean without any isotopic exchange with the atmosphere, and with no contributions from the well equilibrated surface waters, the new deep waters would have a $\delta^{13}\text{C}_{\text{as}}$ signature identical to the upwelled deep waters formed elsewhere (that is, the North Atlantic). Because of the extensive entrainment of subsurface waters during deep water formation, today Southern Ocean source waters ($\delta^{13}\text{C}_{\text{as}} = 0.2\text{‰}$) are only slightly enriched from the upwelled North Atlantic deep water value ($\delta^{13}\text{C}_{\text{as}} = -0.4\text{‰}$) as compared with the surface water ($\delta^{13}\text{C}_{\text{as}} = 1\text{‰}$). In the glacial ocean, deep water renewal in the Southern Ocean could have either modified the $\delta^{13}\text{C}_{\text{as}}$ of the upwelled water towards more positive values, or, in the other extreme case, not modified the $\delta^{13}\text{C}_{\text{as}}$ of incoming water at all. Thus, the low $\delta^{13}\text{C}_{\text{as}}$ signature we find in the dense portions of the glacial oceans cannot have been generated in the Southern Ocean, but must have been imported from elsewhere.

Where did the waters in the Southern Ocean and the deep Pacific get their low $\delta^{13}\text{C}_{\text{as}}$ signature if not in the Antarctic? Contributions from, and exchange with, both Southern Ocean surface waters and GNAIW would have drawn the deep Pacific/Antarctic $\delta^{13}\text{C}_{\text{as}}$ to positive values. We are left with the need for a source of glacial Pacific deep water (GPDW), the Pacific being the only other part of the glacial ocean showing the low $\delta^{13}\text{C}_{\text{as}}$ end-member values. Glacial Cd values in the deep eastern equatorial Pacific were considerably higher than in the northwest Pacific. This enhanced ventilation on the western side of the ocean basin is analogous to today's Atlantic, where the waters in the western basin are replenished more quickly by the newly formed North Atlantic deep waters from the north, and Antarctic bottom waters from the south, supporting the existence of GPDW.

The presence of GPDW has been suggested previously, but has not been widely accepted primarily because of the low $\delta^{13}\text{C}$ values in the deep glacial Pacific. A high nutrient content was inferred from these data without regard to the potentially large effects of air-sea exchange on $\delta^{13}\text{C}$ (refs 6, 7). But an intermediate water source (1–2 km) for the glacial Pacific has been postulated, based on the relatively high $\delta^{13}\text{C}$ values and inferred low nutrient concentration^{3,8}. In contrast, the study reported here suggests that, when allowance is made for the effects of both biological cycling and air-sea exchange, the $\delta^{13}\text{C}$ values in the deep glacial Pacific are not inconsistent with a GPDW source, and when combined with the planktonic data from the Southern Ocean in fact suggest a GPDW source.

Other studies have also supported the presence of GPDW, based on sedimentological evidence for bottom-water flow⁹, changes in the redox conditions of North Pacific sediments¹⁰, increased calcite preservation^{11,12} and low Cd concentrations in the glacial deep Pacific⁵. An ocean model shows that the atmospheric circulation patterns generated by atmospheric models using last glacial maximum boundary conditions increase surface salinity in the northwest Pacific enough to induce deep convection¹⁷.

What information about the glacial ocean can we glean from the end-member $\delta^{13}\text{C}_{\text{as}}$ values themselves? By definition, $\delta^{13}\text{C}_{\text{as}}$ of average deep ocean water was 0‰. If all deep water sources had similar temperatures and initial nutrient concentrations, positive $\delta^{13}\text{C}_{\text{as}}$ means only that this source was exposed to air-sea exchange processes which led to more complete isotopic equilibration of the oceanic carbon with the atmosphere at cold temperatures than the other deep water sources; a negative $\delta^{13}\text{C}_{\text{as}}$ means only that this water mass was less well equilibrated than other deep sources when it left the surface. Only by consid-

ering the $\delta^{13}\text{C}$ of atmospheric CO_2 along with the mean ocean changes in $\delta^{13}\text{C}$ can we determine in an absolute sense whether there was increased equilibration of the glacial deep water masses with the atmosphere. There is evidence that the $\delta^{13}\text{C}$ of the glacial atmosphere was no different¹⁸ or only slightly (1‰) lighter¹⁹ than today, indicating that deep water, on average, was equilibrated with the glacial atmosphere to about the same degree as it is today. It is important to note that today, high-latitude surface water $\delta^{13}\text{C}$ is out of equilibrium with atmospheric carbon by up to 2‰ (refs 2, 20). A change in the residence times, temperature history or the biological history of the waters that eventually contribute to the sinking deep waters could easily cause the range of $\delta^{13}\text{C}_{\text{as}}$ values observed in the glacial ocean. The GNAIW with their high $\delta^{13}\text{C}_{\text{as}}$ may have reflected the closed-loop circulation in the North Atlantic, with the constant re-exposure of its waters to the atmosphere at low temperatures²¹. By analogy to today's low- $\delta^{13}\text{C}_{\text{as}}$ Atlantic source, the low- $\delta^{13}\text{C}_{\text{as}}$ GPDW may also have reflected a warm surface water history of the contributing waters with insufficient time for these waters to re-equilibrate isotopically with the atmosphere at low temperatures.

Many uncertainties remain about the reliability of benthic foraminifera as recorders of nutrient and $\delta^{13}\text{C}$ distributions in the ocean. For example, Mackensen *et al.*²² find that modern foraminiferal $\delta^{13}\text{C}$ can be significantly lower than ambient bottom water in the Southern Ocean,²² and Boyle⁵ finds inexplicably low Cd concentrations in North Pacific core tops. As our understanding of how oceanic $\delta^{13}\text{C}$ and Cd are recorded in benthic foraminifera improves, the specific conclusions about glacial ocean circulation reached here may well change; but the extraction of the air-sea exchange signature, a conservative tracer, from $\delta^{13}\text{C}$ measurements provides a powerful palaeoceanographic tool. When glacial $\delta^{13}\text{C}$ and Cd estimates⁵ are interpreted literally as the glacial deep-ocean values, a coherent picture of the glacial ocean circulation can be seen. These tracers indicate at least two distinct sources of deep water to the glacial ocean, one less-dense source originating in the North Atlantic with a high $\delta^{13}\text{C}_{\text{as}}$ signal, and one more-dense source with a low $\delta^{13}\text{C}_{\text{as}}$ signal which may have formed somewhere in the North Pacific. There is no reason to believe that deep water did not upwell in the Antarctic, densify and sink as deep waters of Antarctic origin but this process left no distinct $\delta^{13}\text{C}_{\text{as}}$ signature. □

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A hominid tibia from Middle Pleistocene sediments at Boxgrove, UK

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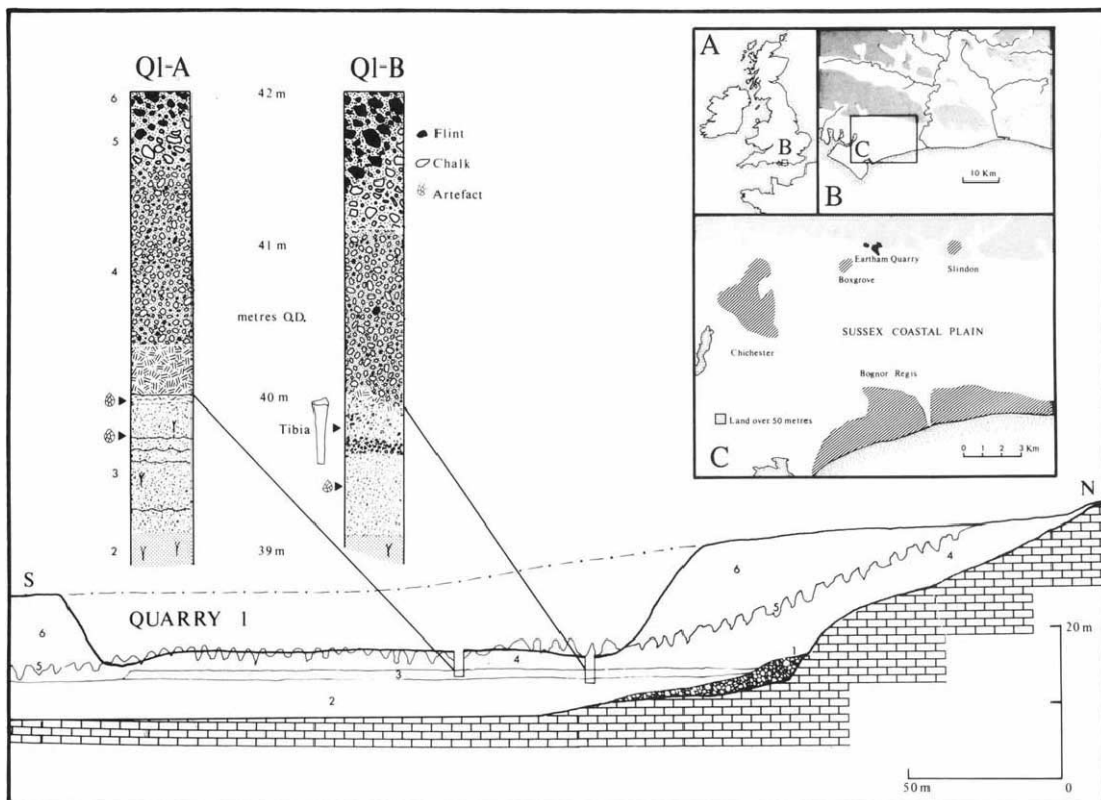
FOSSIL hominids from the earlier Middle Pleistocene of Europe are very rare and the Mauer mandible is generally accepted as the most ancient, with an estimated age of 500 kyr^{1,2}. We report here on the discovery of a human tibia, in association with stone tools, from calcareous silts at the Lower Palaeolithic site of Boxgrove, West Sussex, UK^{3,4} (Fig. 1). The silt units are correlated by mammalian biostratigraphy to an, as yet unnamed, major temperate stage or interglacial that immediately pre-dates the Anglian cold stage⁵. Accordingly, the temperate sediments are equated with oxygen isotope stage 13 (ref. 6) and are therefore roughly coeval with the Mauer mandible. The massive tibia is the oldest hominid fragment from the British Isles and provides the first information about the manufacturers of the early Acheulian industries of Europe. It is assigned to *Homo cf. heidelbergensis*.

The Boxgrove Project was begun in 1985 to excavate areas of Middle Pleistocene land surfaces under threat from quarrying.

Preliminary analyses of the stratigraphy³ had revealed evidence of human activity at all levels throughout the sequence. This activity was particularly concentrated at two discrete horizons within and on the surface of a lagoonal silt unit (Fig. 1). The project was run on a multidisciplinary basis to ensure that the archaeological material, which consists of flint bifaces (Fig. 2), the debitage from their manufacture and butchered animal remains, was placed in an accurate temporal and palaeoenvironmental context. At the end of 1993 a hominid tibia was excavated from calcareous silts⁷ laid down by a spring, originating at the old cliff line (Fig. 1). These silts are higher-energy correlatives of the upper part of the Slindon Silts and their associated soil horizon found elsewhere at the site.

The tibia is from the left side, but lacks its proximal and distal ends, with a preserved posterior length of 294 mm (Fig. 3a). A study of the morphology of the bone shows that there is a medially placed nutrient foramen, a well marked soleal line and a particularly prominent vertical line. Using these landmark features and by comparison with Neanderthal tibiae from Spy, Kiik-Koba, La Chapelle and Tabun, it is possible to calculate that the Boxgrove tibia must have exceeded 355 mm in total length. It is broken near its midshaft and the cross-section shows very thick cortical bone (Fig. 3b). The dimensions of the bone near the midshaft are very large (Table 1) and even the minimum circumference, taken distally, approximates the Neanderthal midshaft maximum. Using Hartwig-Scherer's⁸ predictor from tibial midshaft circumference, body weight is estimated at greater than 80 kg. Given the lack of comparative material from the earlier Middle Pleistocene and pending description of unpub-

FIG. 1 Map showing the location of Boxgrove and composite section through the geology in quarry 1, including detail of deposits containing the hominid tibia. Key to the section diagram is as follows: 1, Raised beach gravels; 2, Slindon Sands; 3, Slindon Silts, with soil horizon developed in upper part at Q1-A; 4, Calcareous silts and gravels, derived from weathering of the chalk cliff; 5, Solution weathering contact; 6, Soliflucted Tertiary regolith. The site at Boxgrove is situated at the foot of the South Downs, 7 km east of Chichester (see inset). The pre-Pleistocene geological framework³⁻⁵ at the site was modified by a Middle Pleistocene marine transgression that cut back into the dip slope of the downland block. Coastal processes associated with the high sea-level transgression resulted in the formation of a chalk cliff and wave-cut platform, similar



to modern analogues seen around the coastline of southern and south-eastern England today. The sediments of the Slindon Formation were deposited during this period of marine transgression. As sea level dropped, a soil horizon developed on the surface of the Slindon Silts^{7,16}. The period of soil formation lasted for probably no more than 100 years before the land-surface was flooded again, this time by fresh water. The result of this inundation was the creation of an alder/fen carr, and this marshy environment was subsequently choked with sediments that were washed off the downland block. These colluvial processes are the first signs of climatic deterioration and are thought to coincide with

devegetation and increased precipitation. Changes in the mammalian fauna are also apparent within these sediments. There is a marked decline and eventual disappearance of true interglacial indicator species such as *Clethrionomys glareolus*, *Muscardinus avellanarius* and *Apodemus sylvaticus*¹⁷ that are associated with mixed oak woodland, and an increase in more open country forms such as *Clethrionomys rufocanus*, *Microtus oeconomus* and *Lemmus lemmus*. The next suite of deposits, mass movement gravels brought downslope by solifluction, attest to the onset of full periglacial conditions.

TABLE 1 Boxgrove tibia dimensions

	Midshaft circumference	Midshaft antero-posterior diameter	Midshaft medio-lateral diameter	Distal minimum circumference
Boxgrove	105	39.5	30	96.5
KNM-ER 803	80	28.6	20	71
Zhoukoudian	78	27	21	—
Ngandong	84–101	29.5–37	21.5–27	82
	(n = 2)	(2)	(2)	(1)
Sambungmachan	86	32	21.2	76
Broken Hill E.691	91.5	34	24	80
Atapuerca	76–80	27.6–29.3	20–22.5	67–84
	(n = 2)	(2)	(2)	(2)
Neanderthal	75–98	27–38.4	20–25.8	70–87
	(n = 12)	(13)	(13)	(4)
Up. Palaeolithic	74–100	27–39	17–24	62–89
	(n = 6)	(6)	(6)	(7)
Modern	69.5–101.3	19.5–34.3	17–30	53–83
	(n = 23)	(100)	(100)	(99)

Midshaft measurements taken immediately below the break. Comparative data from Endo and Kimura¹⁰, Day and Leakey¹¹, Santa Luca¹², Trinkaus¹³, Baba et al.¹⁴, Arsuaga et al.¹⁵, Hartwig-Scherer⁸, and this study.

lished samples from sites like Atapuerca or further discoveries, the bone can only be assigned to *Homo cf. heidelbergensis*⁹, from the holotype defined on a human mandible discovered in the lower part of the Mauer Sands in 1907. However, it clearly indicates the exceptional skeletal robustness of the first individual directly associated with the early Acheulian industries of Britain and Europe.

The sedimentary sequence at Boxgrove, described in Fig. 1, spans a major warm climate cycle, an interglacial, into the ensuing cold stage or glacial. Dating this interglacial/glacial cycle has, in the absence of any reliable absolute dates, been undertaken using correlative mammalian biostratigraphy. It can be demonstrated on the basis of the evolutionary transition of the water vole *Mimomys savini* to *Arvicola terrestris cantiana*^{17,18}, that the fauna at Boxgrove post-dates the Forest Bed fauna¹⁹ at West Runton, the type site of the Cromerian²⁰. The Boxgrove mammalian fauna has many affinities with the type Cromerian. It contains species such as the soricids, *Sorex savini* and *Sorex runtonensis*; the rodents, *Pliomys episcopalensis* and *Microtus gregalis* ('*gregaloides*' morphotype); the bear *Ursus deningeri* and the rhinoceros *Stephanorhinus hundsheimensis*, all of which are absent from sites such as Swanscombe^{19,21} and Barnham²², which immediately post-date the Anglian glaciation. This glaciation is correlated with oxygen isotope stage 12, whereas at West Runton the type Cromerian sediments underlie the glacial deposits of the Anglian. The exact position of West Runton in the British

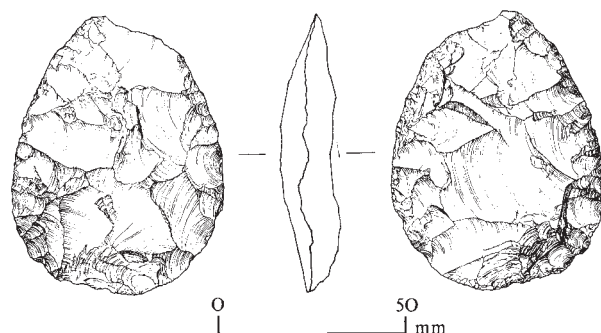


FIG. 2 Typical flint biface from the soil horizon, developed on the surface of the Slindon Silts.

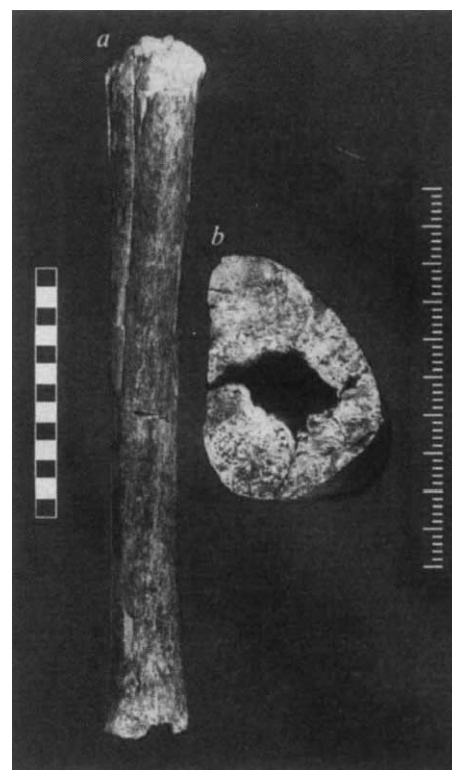


FIG. 3 a, Anterior view of the Boxgrove tibia. b, Cross-section of Boxgrove tibia below the midshaft break. Anterior surface at top of figure, viewed distally. The morphology of the tibia will be compared in detail with other specimens from the European Middle and Late Pleistocene using computed tomography. Detailed excavations of the calcareous spring deposits will take place in 1995, with the expectation that further hominid material will be discovered. Scale bar, 1 cm.

Pleistocene sequence is not known; however, it must post-date the Matuyama–Brunhes boundary²³ as the sediments are normally magnetized. The period of ~300 kyr between the beginning of the Brunhes Epoch and the Anglian contains four major temperate stages and three major cold stages in the oceanic record^{6,24}. In Britain these stages are best represented lithostratigraphically in the terrace deposits of the pre-diversion course of the River Thames^{25–27}. Mapping of terraces has demonstrated that there is one major temperate episode²⁸ between the Cromerian fauna at Little Oakley²⁹ and the diversion of the Thames into its present valley by the ice of the Anglian glaciation. Thus the best fit for the Boxgrove interglacial sediments would be in oxygen isotope stage 13, between 524 and 478 kyr³⁰. This correlation fits well with dates from sites with similar faunal assemblages in mainland Europe such as Nordbergum³¹ in the Netherlands, Kärlich G (soil horizon)³² and Miesenheim in the Rhine valley³³, and Mauer¹ in southern Germany. These sites are all assigned to Interglacial IV of the Cromerian Complex²⁴. In Britain, sites thought to be of the same age as Boxgrove are found at Ostend in Norfolk³⁴, Westbury-sub-Mendip in Somerset^{35,36} and High Lodge in Suffolk³⁷. Further work on the Boxgrove tibia and the area where it was discovered is planned for 1994/95. □

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Direct measurement of attentional dwell time in human vision

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In vision, attentional limitations are reflected in interference or reduced accuracy when two objects must be identified at once in a brief display^{1,2}. In our experiments a brief temporal separation was introduced between the two objects to be identified. We measured how long the first object continued to interfere with the second, and hence the time course of the first object's attentional demand. According to conventional serial models, attention is assigned rapidly to one object after another, with a dwell time of only a few dozen milliseconds per item^{3,4}. But we report here that interference lasts for several hundred milliseconds—an order of magnitude more than the prediction of conventional models. We suggest that visual attention is not a high-speed switching mechanism, but a sustained state during which relevant objects become available to influence behaviour. This conclusion is consistent with recent physiological results in the monkey⁵.

Conventional estimates of attentional dwell time are based on measurements of the speed^{3,6} or accuracy^{4,7} of visual search. If the time taken to detect a target stimulus increases by a few milliseconds for each nontarget added to a display, one interpretation is that attention moves rapidly and serially from one object to another until the target is found^{3,6}. Corresponding arguments concern accuracy when displays are brief⁴. However, such data can also be explained by limited-capacity parallel processing models with substantially greater dwell times^{8–10}. According to these models, attention can be divided between several objects at once, but with increasing cost as the number of attended objects increases.

To address this ambiguity, we adapted the techniques of visual search to measure directly how long an object that must be identified continues to occupy attentional capacity. As with standard search techniques, we presented several display items at unpredictable locations within a single trial. But rather than presenting display items simultaneously, we presented just two items separated in time. Attention was engaged on the first object by asking for its identification. At varying intervals afterwards, the second object was presented and interference of the first

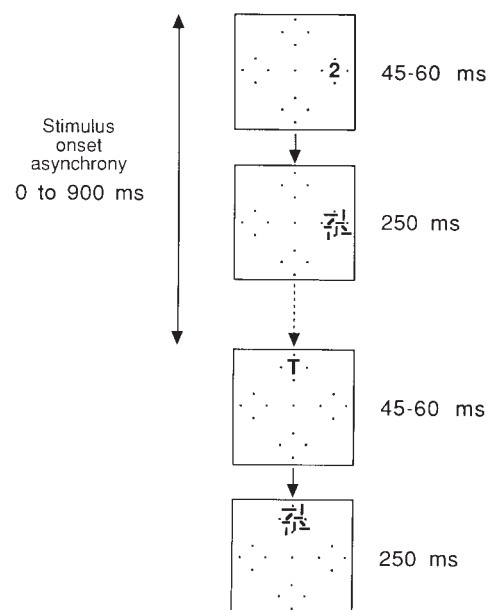


FIG. 1 Events on a single trial. A $5^\circ \times 5^\circ$ fixation display, present at all times, consisted of a central dot and four frames indicating possible stimulus positions. Subjects initiated the trial by pressing a key; one character was presented after a random delay of 0–500 ms, and a second after a further delay of 0–900 ms, measured from onset to onset (stimulus onset asynchrony, or SOA). One of these characters was a green digit (2 or 5), appearing in one of the horizontal locations (left or right frame). The other was a red letter (L or T), appearing in one of the vertical locations (top or bottom frame). The order of these two was unpredictable. Characters measured $0.5^\circ \times 0.6^\circ$, and were presented for 45–60 ms (average 57 ms), as determined for each subject in an initial series of at least 60 calibration trials. Each character was immediately followed by a 250-ms masking pattern used to limit visual persistence. In a testing session, there were three blocks of 156 trials each, given in counterbalanced order. These corresponded to three types of reports: subjects were instructed to identify just the green digit (ignore vertical locations), just the red letter (ignore horizontal locations), or both characters. Subjects pressed keys to indicate their responses, and responses were always withheld until both characters had been presented. Feedback (overall percent correct) was given at the end of each block. Nine subjects (mean age 33) participated in two sessions each.

object on the second was measured as a function of temporal separation. This method tracked continuously in time the attentional demands of first object identification.

On each trial of our first experiment (Fig. 1), two alphanumeric characters were presented, a green digit and a red letter. Each character was presented for only 45–60 ms, with an unpredictable interval of 0–900 ms (measured from onset to onset) between the two. When both characters were to be identified (Fig. 2; filled circles), the one coming second suffered prolonged interference. Indeed, at separations of 100–300 ms, the second character was identified even less accurately than characters presented simultaneously. This should be expected if attention is divided equally between characters with simultaneous presentation, but focused on the first character when presentation is asynchronous. Interference gradually declined at separations greater than 300 ms. In contrast, performance was independent of temporal separation when only one character was identified and the other ignored (Fig. 2; open circles). Measured directly by this method, identifying an object occupies attention for at least several hundred milliseconds.

To rule out the possibility that this estimate of attentional dwell time might depend upon the requirement to make independent, overt responses to each character, we adapted our methods more closely to visual search, so that the task was simply to detect the presence of a target among non-targets. The two characters presented on each trial were both Ls, presented either upright (target) or rotated 90° clockwise or anticlockwise (non-targets). After each trial the observer indicated with a single response whether a target had been seen. Results were very much as before. When the first character was a non-target, a subsequent target suffered prolonged interference (Fig. 3).

A different aspect of attentional dynamics is the time taken to respond to a cue indicating how attention should be directed: for example, which items in a display should be reported^{11,12}. Such measurements reflect the time needed to identify the cue, to determine its meaning, and to establish the appropriate selec-

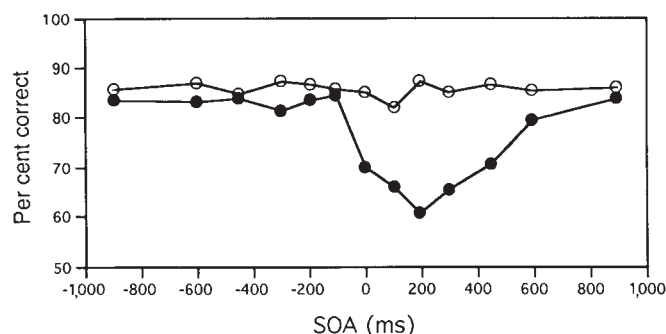


FIG. 2 Results from experiment 1. The figure shows accuracy of target identification either when both characters were attended (filled circles) or when one could be ignored as it appeared in an irrelevant location (open circles). Negative SOAs refer to the item presented first in the trial and positive SOAs refer to the item presented second. Performance varied little between testing sessions, or on letters versus digits; the figure averages over these variables. When both characters were attended, the second suffered interference at temporal separations at least up to 450 ms. As compared to asymptote (estimated by mean accuracy at maximal temporal separation), accuracy was significantly reduced at each SOA between 0 and 450 ms ($P < 0.004$ or better in each case, t -test). In this experiment there was no interference from a stimulus that could be ignored (conditions in which subjects ignored either horizontal locations (green digits) or vertical locations (red letters)). For these conditions (open circles), data for the single character identified are plotted as a function of SOA with respect to the ignored character. In other experiments mild interference sometimes remains in this case, suggesting that even a character in an ignored location has some tendency to attract attention to itself¹⁸.

tion rule. In our experiments there was no instructional cue to be interpreted. As in conventional visual search, the subject simply had to identify the separate display items. We have shown that processing a single item in a visual search or identification task occupies attentional capacity for several hundred milliseconds.

In further experiments we obtained similar estimates of dwell time under a variety of different conditions. Increasing the complexity and duration of response processes for the first object had no effect, differentiating the interference measured here from delays in response processing known by convention as the 'psychological refractory period'¹³. We also obtained similar estimates of dwell time when objects appeared successively at fixation instead of at different spatial locations. The time we measured is concerned not with shifting attention from one location to another in the visual field, but with how long an object that must be identified continues to occupy attentional capacity. Indeed, an extended attentional dwell time helps explain the results of previous experiments using rapid sequences of many characters, presented one after the other at a single location^{14,15}. Finally, if the first display contained two relevant characters rather than one, interference with the second display was strengthened, reflecting increased division of attentional capacity.

Conventional visual search results can be explained either by high-speed serial models or limited-capacity parallel models of visual attention. By measuring attentional dwell time directly, we have shown that high-speed serial models are incorrect. Instead, we suggest attention to a relevant object is a sustained

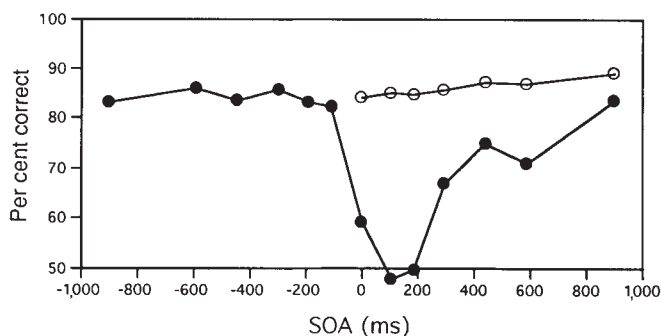


FIG. 3 Results from experiment 2. Two characters were presented as before, one in the left or right display frame and one in the top or bottom frame. Stimulus exposure durations ranged from 40–104 ms (average 73 ms); SOAs and other details were as before. Each character was a letter L, measuring $0.6^\circ \times 0.6^\circ$, presented either in its normal upright orientation (target) or rotated 90° clockwise or anticlockwise (non-targets). A single target, either the first or the second character to appear at random, was present on 50% of the trials. The subject's task was to indicate whether or not a target had appeared by pressing a key at the end of the trial. Data presented come only from a condition in which both characters were attended, because the target could appear in any display location. For target-present trials (filled circles), data have been separated into negative (target presented first) and positive (target presented second) SOAs. Target detection was substantially impaired by a preceding non-target, up to temporal separations of at least 300 ms (differences from asymptotic accuracy significant at each SOA between 0 and 300 ms, $P < 0.001$ in each case), and probably longer ($P < 0.06$ at SOA of 450 ms; $P < 0.02$ at SOA of 600 ms). For trials with no target, SOAs cannot be defined as negative or positive; data (open circles) are shown simply as a function of temporal separation between the two non-targets. For these trials, accuracy was virtually independent of SOA, showing that interference at short SOAs was reflected almost entirely in missed targets rather than false positives. As before, conditions in which one character could be ignored, because its display location was known to be irrelevant, showed much attenuated interference (data not shown). Data come from 24 subjects (mean age, 34), each participating in a single session.

state, during which that object is available to awareness for the control of behaviour. Although largely independent of eye movements¹⁶, attention has a comparable dwell time. This conclusion is consistent with recent neurophysiological data. In the high-level visual cortex of the monkey, attention is reflected in part by suppression of neuronal responses to ignored objects^{5,17}. This suppression develops over several hundred milliseconds (ref. 5), as does the interference with subsequent inputs shown here in behaviour. □

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Translational regulation of *nanos* by RNA localization

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LOCALIZATION of the maternally synthesized *nanos* (*nos*) RNA to the posterior pole of the *Drosophila* embryo provides the source for a posterior-to-anterior gradient of Nos protein^{1,2}. Correct spatial regulation of *nos* activity is essential for normal pattern formation. High local concentrations of Nos protein in the posterior of the embryo are necessary to inhibit translation of the transcription factor Hunchback in this region^{3,4}, and thus permit expression of genes required for abdomen formation (see ref. 5 for review). By contrast, misexpression of Nos protein at the anterior of the embryo prevents translation of the anterior morphogen Bicoid, suppressing head and thorax development^{1,6–9}. Posterior localization of *nos* RNA is mediated by sequences within the *nos* 3' untranslated region (3'UTR)¹ and requires the function of eight genes of the 'posterior group'^{2,6}. Although the unlocalized *nos* RNA is stable in embryos from females mutant for any of the posterior group genes, these embryos appear to lack *nos* activity because they develop the abdominal defects characteristic of embryos produced by *nos* mutant females^{2,6,10–14}. We report here that unlocalized *nos* RNA is translationally repressed. Translational repression is mediated by the *nos* 3'UTR and can be alleviated either by replacement of the 3'UTR with heterologous 3'UTR sequences or by posterior localization. Thus, RNA localization provides a novel mechanism for translational regulation.

nos RNA localization requires the activity of at least eight posterior group genes including *oskar* (*osk*) and *vasa* (*vas*)². Embryos produced by females mutant for these genes fail to localize *nos* RNA but contain wild-type levels of this RNA² (Fig.

1a). To determine whether the lack of *nos* function in these embryos results from failure to generate a concentrated source of Nos protein or from lack of Nos protein altogether, we analysed Nos protein in extracts of embryos from wild-type and mutant females. Nos protein was not detected in extracts of embryos from *osk*[−] or *vas*[−] females (Fig. 1b). Thus, the similarity of phenotype between embryos from *osk*[−] or *vas*[−] females and embryos from females carrying a null *nos* mutation results from the lack of Nos protein. This suggests that unlocalized *nos* RNA is translationally inactive and that this translational inhibition is relieved by RNA localization. A link between RNA localization and translation is further supported by the finding that an increase in the *osk* gene dosage from 2 copies (wild-type females) to 4 copies (4 × *osk*⁺ females) results in an increase both in the amount of *nos* RNA localized to the posterior of the embryo^{15,16} and in the amount of Nos protein produced (Fig. 1b), whereas the total *nos* RNA level remains constant (Fig. 1a). This suggests that *osk* is limiting for *nos* RNA localization and translation.

Because *nos* RNA localization depends on the *nos* 3'UTR, we tested whether translational regulation of *nos* RNA is also mediated through 3'UTR sequences. To determine whether translational repression of unlocalized *nos* RNA requires the *nos* 3'UTR, we constructed a hybrid gene in which the *nos* 3'UTR

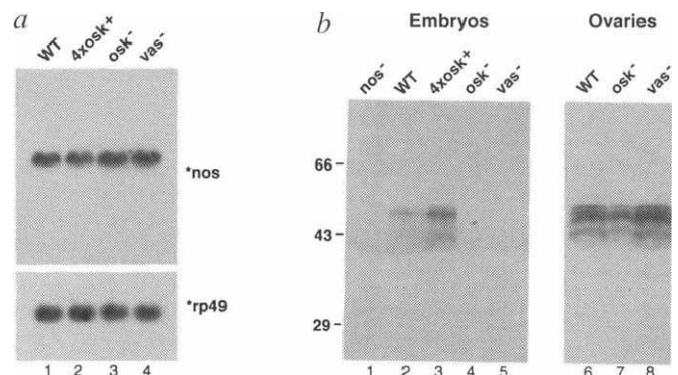


FIG. 1 Effects of posterior group genes on *nos* RNA and protein levels. **a**, Northern blot analysis of total RNA from 0–2 h embryos from females of the following genotypes: WT (wild-type); 4 × *osk*⁺ (4 copies of the wild-type *osk* gene¹⁵); *osk*[−] (*osk*⁵⁴/*osk*⁸⁴; refs 11, 22), *vas*[−] (*vas*^{PD}/*vas*^{D1}; refs 6, 12). The blot was hybridized sequentially with probes for *nos* RNA (**nos*) and for rp49 RNA (*rp49) for standardization. Phosphorimage quantification of the blot revealed that equivalent amounts of *nos* RNA are present in embryos of each genotype. **b**, Immunoblots of extracts from 0–2-h embryos and ovaries. Equivalent amounts of extract of each genotype, as determined by Ponceau-S staining of the blots, were fractionated on 10% SDS–polyacrylamide gels. Nos protein was detected with an anti-Nos polyclonal antibody². No immunoreactive material is present in embryos from null *nos*[−] females (*nos*^{BN}; ref. 2). Nos protein appears as a set of bands in embryos and ovaries from WT females. The same bands are present in ovaries from *osk*[−] and *vas*[−] females but not in embryos from these mutants. Embryos from *tudor*[−] and *valois*[−] females show reduced amounts of Nos protein (data not shown); the mutant alleles of these genes are most probably not null, however, and permit some *nos* RNA localization². Relative molecular mass markers: BSA, 66K; ovalbumin, 43K; carbonic anhydrase, 29K. **METHODS.** **a**, Northern blotting as in ref. 1, using 5 µg total RNA. ³²P probes were synthesized from the pN5 cDNA (*nos*; ref. 17) and the ~600 bp *EcoRI*–*HindIII* fragment of HR0.6 (rp49; ref. 23). **b**, Ovaries from virgin females fed for 2 days at 25 °C were dissected into EBR²⁴ and washed with PBS. 0–2 h embryos were collected at room temperature, dechorionated with 50% Chlorox, and washed with dH₂O. Both tissues were frozen in liquid N₂ and stored at −80 °C. Frozen tissues were homogenized in boiling sample buffer (0.125 M Tris–HCl pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 5M urea) and boiled for 3 min. Supernatants were separated by SDS–PAGE followed by transfer to nitrocellulose. Immunoblotting with the anti-Nos antibody according to ref. 25, supplementing with 5% calf serum.

was replaced by sequences from the α -tubulin 3'UTR (*nos-tub3'UTR*; Fig. 2a). The transgene, which encodes the *nos-tub3'UTR* hybrid RNA, was then introduced into flies by P element-mediated germ-line transformation. The *nos-tub3'UTR* transgene behaves as a dominant female sterile mutation. Embryos produced by females carrying a single copy of the transgene fail to hatch, lack anterior structures, and often show duplication of posterior structures at the anterior (Fig. 2b-d). This phenotype resembles that produced by mislocalization of *nos* RNA to the anterior of the embryo, where Nos protein then acts to inhibit translation of both maternal *hunchback* (*hb*) and *bicoid* (*bcd*) RNA^{1,6,9}. The *nos-tub3'UTR* transgene produces sufficient *nos* activity to complement completely the *nos*⁻ abdominal defect (Fig. 2d). Furthermore, the suppression of anterior development indicates the presence of substantial *nos* activity in the anterior of the embryo.

Analysis of embryos by *in situ* hybridization confirmed that the transgene produces an unlocalized *nos* RNA (Fig. 2e,f) resulting in uniformly distributed Nos protein (Fig. 2g,h). Expression of Nos protein throughout the embryo results in uniform reduction in the level of maternal Hb protein (Fig. 2i,j). Nos protein now present anteriorly inhibits translation of Bcd protein, leading to a reduction of the *bcd*-dependent domain of zygotic *hb* expression (Fig. 2k,l). Thus, these embryos have sufficient *nos* activity to reduce the levels of Hb and Bcd, allowing abdomen formation and inhibiting formation of anterior structures, respectively. The effect on translation of *bcd* RNA is

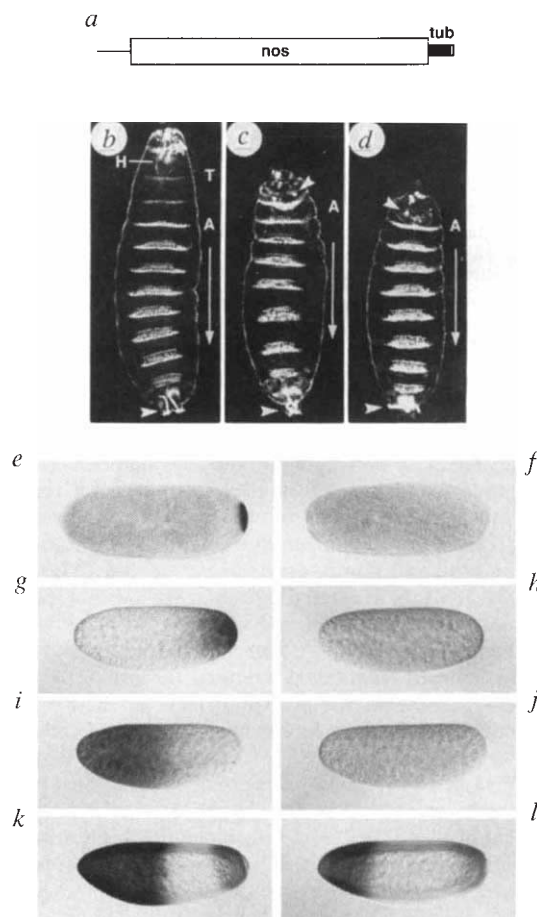
less severe than that produced when *nos* RNA is localized to the anterior of the embryo¹, however, and reflects a lower concentration of *nos* RNA at the anterior of the embryo when it is uniformly distributed than when it is localized to the anterior. Indeed, increasing the dosage of the transgene (see Fig. 2 methods) provides sufficient *nos* to eliminate *bcd* activity (data not shown).

In contrast to the unlocalized *nos* RNA in embryos from *osk*⁻ and *vas*⁻ females, the unlocalized *nos-tub3'UTR* RNA is translationally active (Fig. 3b). A single copy of the *nos-tub3'UTR* transgene produced ~30% as much RNA as the two wild-type *nos* genes (Fig. 3a). Embryos from females bearing only the single transgene (*nos-tub3'UTR*/+; *nos*⁻), however, contain similar amounts of Nos protein as embryos from females with twice the wild-type gene dosage ($4 \times \text{nos}^+$; Fig. 3b). Thus, sequences within the *nos* 3'UTR mediate translational repression of unlocalized *nos* RNA; removal of the *nos* 3'UTR and replacement with heterologous 3'UTR sequences creates an unlocalized *nos* RNA which is translated to produce a uniform distribution of Nos protein.

Translation of *nos-tub3'UTR* RNA does not depend on posterior group genes that control localization-dependent translation of the endogenous *nos* RNA. Nos protein is produced in embryos from females that carry the *nos-tub3'UTR* transgene and are mutant for *osk*, *vas*, *valois*¹² or *tudor*¹⁰. These embryos are indistinguishable from those derived from *nos-tub3'UTR* females, with abdominal segments present and anterior develop-

FIG. 2 Expression of unlocalized *nos* RNA bearing a heterologous 3'UTR. **a**, The *nos-tub3'UTR* hybrid RNA: thin line, *nos* 5'UTR; open box, *nos* coding sequence; filled box, α -tubulin 3'UTR. **b-d**, Larval cuticles. Wild-type (**b**) with head (H), thorax (T), abdomen (A). The posterior tail (telson) contains the light refractile filzkörper (arrowhead). Embryos from transgenic females (*nos-tub3'UTR*/+) (**c**) and from transgenic females mutant for *nos* (*nos-tub3'UTR*/+; *nos*⁻) (**d**) are indistinguishable and show loss of head and thorax. Filzkörper are often duplicated at the anterior (arrowhead). Arrows indicate anterior-posterior polarity. Ventral view, anterior up. **e, g, i, k**, Embryos from wild-type and **f, h, j, l**, from *nos-tub3'UTR*/+; *nos*⁻ females. Because the *nos* allele used, *nos*^{BN}, lacks *nos* RNA in embryos², the latter embryos contain only the hybrid *nos-tub3'UTR* RNA. **e, f**, Whole-mount *in situ* hybridization to embryos using an antisense *nos* RNA probe. Unlike the wild-type *nos* RNA, *nos-tub3'UTR* RNA appears uniformly distributed throughout the embryo. Comparison of embryos hybridized with antisense, sense, and non-specific RNA probes demonstrates that some *nos* RNA remains uniformly distributed in wild-type embryos (data not shown). **g, h**, Whole-mount staining of embryos with anti-Nos antibody. Nos protein is uniformly distributed in embryos from *nos-tub3'UTR* females. **i, j**, Whole-mount antibody staining with anti-Hb antibody. At nuclear cycle 8, the antibody detects an anterior-posterior gradient of Hb protein produced from the maternal *hb* RNA in embryos from wild-type females. This protein is reduced to low levels throughout embryos from *nos-tub3'UTR* females. At syncytial blastoderm the anti-Hb antibody detects Hb protein produced from the zygotic *hb* transcript (**k, l**). Because the anterior domain of zygotic *hb* expression is controlled by *bcd*, reduction of this domain of Hb expression in *nos-tub3'UTR* embryos reflects an effect on *bcd* expression. Anterior left, dorsal up.

METHODS. **a**, The *nos-tub3'UTR* transgene was constructed by replacing the 834 bp *EcoRI*-*XhoI* *nos* 3'UTR fragment of pHSXgnosb^R (ref. 1) with the 197 bp *SalI*-*HpaI* α -1 tubulin 3'UTR fragment after end-filling²⁶. A haemagglutinin epitope tag²⁷ was inserted at the 5' end of the *nos* coding region (D. Curtis and R.L., unpublished methods). The *nos-tub3'UTR* hybrid gene was injected into *ry*⁻ embryos for P element-mediated germ-line transformation²⁸. Ten independent lines were analysed for cuticular phenotypes and three of these by whole-mount techniques. Females from two independent lines carrying multiple unlinked insertions produced embryos with two abdomens and telsons in mirror image symmetry. This phenotype is characteristic of embryos lacking *bcd* and *hb*^{1,5}. **b-d**, Dark-field optics. Larval cuticles were prepared as described²⁹. **e-l**, Nomarski optics. Whole-mount *in situ* hybridization with a digoxigenin-labelled antisense probe synthesized from the *nos* cDNA pN5 and whole-mount antibody staining as in ref. 1.



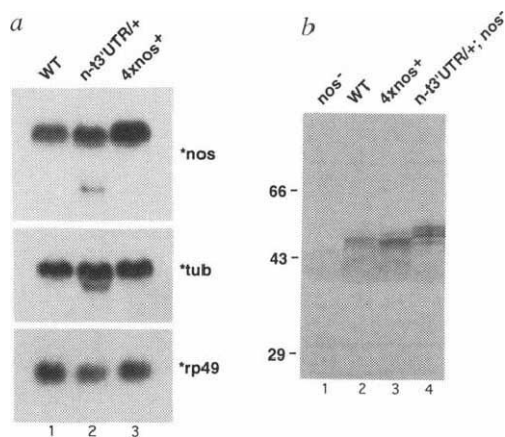


FIG. 3 Removal of the *nos* 3'UTR permits translation of unlocalized *nos* RNA. **a**, Northern blot analysis of RNA from 0–2-h embryos from females of the following genotypes: WT (wild-type), *n-t3'UTR/+* (*nos-tub3'UTR/+*; these embryos contain both wild-type *nos* and *nos-tub3'UTR* hybrid RNAs), *4 × nos*⁺ (4 copies of the wild-type *nos* gene)¹. A probe for *nos* sequences (**nos*) detects both the endogenous *nos* RNA and the *nos-tub3'UTR* RNA, which is 627 nucleotides shorter. A probe for α -tubulin 3'UTR sequences (**tub*) detects the *nos-tub3'UTR* RNA and the endogenous α -tubulin RNA. Quantification showed that a single copy of the *nos-tub3'UTR* transgene produces 30% as much as RNA as do the two copies of the wild-type *nos* gene. **b**, Immunoblotting of extracts from 0–2-h embryos analysed in **a** except that *nos-tub3'UTR* embryos also lack the wild-type *nos* gene (*n-t3'UTR/+; nos*[−]). Thus, only protein synthesized from the hybrid RNA is present in these embryos. Equivalent amounts of extract of each genotype, as determined by Ponceau-S staining, were fractionated on a 10% SDS-polyacrylamide gel. The anti-Nos antibody detects high levels of Nos protein produced by the *nos-tub3'UTR* RNA. All forms of this protein migrate slightly more slowly than do those of the wild-type *nos* gene (compare lanes 2 and 4) as a result of the epitope tag (E.R.G., D. Curtis, and R.L., unpublished data). The tag has no other detectable effect, as a *nos-tub3'UTR* construct lacking the tag behaves identically with respect to its function in embryos and the amount of protein it produces. *M*, markers as in Fig. 1. **METHODS.** **a**, Northern blot analysis as in Fig. 1. The ³²P-labelled α -tubulin probe was synthesized from the 197 bp *ScaI*–*HpaI* fragment of α -1 tubulin. **b**, Immunoblotting as in Fig. 1.

ment suppressed (data not shown). Because Nos protein is active in the absence of these posterior group genes, they must be required primarily for *nos* RNA localization and not for Nos protein function.

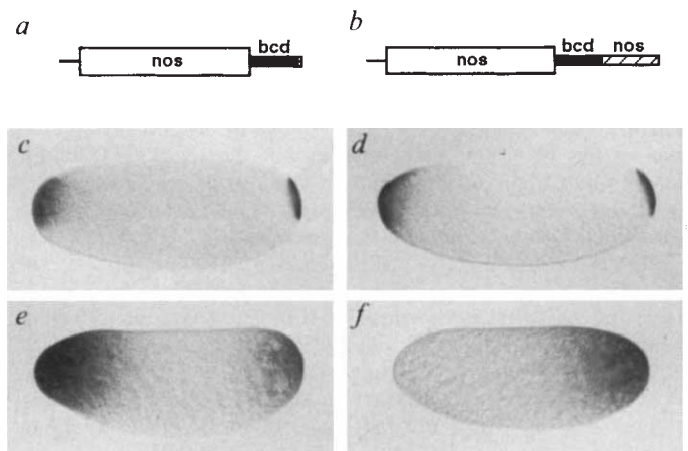
Our experiments suggest that localization-dependent translational regulation provides a mechanism to ensure the restriction of *nos* activity to the posterior of the embryo. Embryos from wild-type females contain both localized and uniformly distributed populations of *nos* RNA (Fig. 2e). In our model, the unlocalized *nos* RNA in embryos from wild-type females is translationally repressed by 3'UTR sequences. It is possible that the 3'UTR sequences of unlocalized *nos* RNA are translationally incompetent, because of their configuration or failure to associate with the translational machinery. Posterior localization mediated by posterior group gene products would alter this configuration or association, allowing translation to occur. Alternatively, translation of the RNA may be actively prevented by factors that interact with the 3'UTR. Interaction of the 3'UTR with the localization machinery would thereby overcome the activity of these factors. This second hypothesis is supported by the finding that *nos* RNA synthesized *in vitro* is translated after injection into embryos¹⁷.

To investigate this question further, we asked whether the *nos* 3'UTR could impose a translational block on an otherwise functional RNA. We have previously shown that replacement of the *nos* 3'UTR by that of the anteriorly localized *bcd* RNA results in localization of this *nos-bcd3'UTR* hybrid RNA (Fig. 4a) to the anterior pole and subsequent production of high levels of Nos protein at the anterior¹ (Fig. 4c, e). When the *nos* 3'UTR is reintroduced, the hybrid RNA containing both the *nos* and *bcd* 3'UTRs (*nos-bcd/nos3'UTR*; Fig. 4b) is localized exclusively to the anterior of the embryo, indistinguishably from the *nos-bcd3'UTR* RNA (Fig. 4d). However, Nos protein is not detected at the anterior of embryos from *nos-bcd/nos3'UTR* females (Fig. 4f) and no phenotype is produced. Thus, localization within the embryo is not sufficient for translation of wild-type *nos* RNA; rather posterior localization is required. Furthermore, this experiment demonstrates that the element(s) within the 3'UTR that mediate repression can act at a distance within the RNA. It is therefore likely that specific factors bind to the 3'UTR of unlocalized *nos* RNA to prevent its translation and that these factors are eliminated or inactivated by components of the posterior localization machinery.

Translational repression of *nos* RNA must occur during the

FIG. 4 The *nos* 3'UTR can repress translation distant from its normal position in the *nos* RNA. **a**, The hybrid RNA in which *nos* 3'UTR sequences are replaced by *bcd* 3'UTR sequences (filled box, *nos-bcd3'UTR*). **b**, The hybrid RNA bearing both *nos* (hatched box) and *bcd* (filled box) 3'UTR sequences (*nos-bcd/nos3'UTR*). **c**, **e**, Embryos from *nos-bcd3'UTR* females. **d**, **f**, Embryos from *nos-bcd/nos3'UTR* females. Whole-mount *in situ* hybridization with an antisense *nos* RNA probe (**c**, **d**) detects both the wild-type *nos* RNA localized to the posterior pole and the hybrid RNAs localized to the anterior pole. A probe for *bcd* sequences showed that both hybrid RNAs are present only at the anterior pole (data not shown). Anti-Nos antibody staining (**e**, **f**) detects Nos protein produced from the wild-type *nos* RNA and the *nos-bcd3'UTR* RNA but not from the *nos-bcd/nos3'UTR* RNA. A transgene in which the *bcd* 3'UTR sequences were added 3' to the *nos* 3'UTR (*nos-nos/bcd3'UTR*) behaved identically to the *nos-bcd/nos3'UTR* transgene (data not shown). Anterior, left; dorsal, up.

METHODS. The *nos-bcd3'UTR* transgene was described previously¹. The *nos-bcd/nos3'UTR* transgene was constructed by inserting the end-filled 627 bp *MluI*–*StuI* *bcd* 3'UTR fragment³⁰ into the *EcoRI* site (end-filled) at the junction of the *nos* coding sequences and 3'UTR in pHSXgnosb^R (ref. 1). Whole-mount *in situ* hybridization and antibody staining as in Fig. 2.



latter stages of oogenesis or following egg activation because Nos protein is present in ovaries from *osk*⁻ and *vas*⁻ females, but not in their embryos (Fig. 1b; see also ref. 6). Furthermore, this result indicates that the *osk* and *vas* products are not absolutely required for *nos* translation. Genes that act to repress the translation of unlocalized *nos* RNA have not been identified. Mutations in such genes should lead to indiscriminate translation of *nos* throughout the embryo and may cause phenotypes similar to those observed in embryos from females carrying the *nos-tub3' UTR* or the *nos-bcd3' UTR*¹ transgenes.

3'UTR-dependent translational repression has been demonstrated for RNAs that are stored in an inactive state before use, either in oocytes or in spermatocytes (see ref. 18 for review), as well as for RNAs whose activity is temporally or spatially restricted during development of somatic tissues (see ref. 19 for review). 3'UTR-dependent RNA localization has also been demonstrated for maternal RNAs in oocytes and embryos as well as for zygotic RNAs in several cell types (see refs 20, 21 for review). *nos* is the first example in which these two processes are linked. □

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Activation of *C. elegans* cell death protein CED-9 by an amino-acid substitution in a domain conserved in Bcl-2

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THE *Caenorhabditis elegans* gene *ced-9* and the human proto-oncogene *bcl-2*, both of which protect cells from programmed cell death, are members of the same gene family^{1–11}. *ced-9* and *bcl-2* were discovered because of the effects of dominant gain-of-function mutations^{12–14}. Such *bcl-2* mutations, which are commonly found in follicular lymphoma, are translocations that result in over-expression of a normal Bcl-2 protein in B cells^{1,13–16}. Here we report that, by contrast, the *ced-9(n1950)* gain-of-function mutation affects the open reading frame of *ced-9* and results in a glycine-to-glutamate substitution in a region highly conserved among all *ced-9/bcl-2* family members. We conclude that this glycine has an important function in *ced-9* regulation, and we suggest that alteration of this glycine in other members of the *ced-9/bcl-2* family might lead to oncogenic activation. We also present genetic evidence suggesting that the CED-9 protein might exist in two distinct forms that have opposite effects on cell death.

To determine the nature of the *ced-9(n1950)* gain-of-function mutation, we probed Southern and northern blots from wild-type and *ced-9(n1950)* worms. No differences were detected on genomic Southern blots (*EcoRI* and *EcoRV* digests) probed with cosmid C41B4 (which contains *ced-9* and about 20 kilobases (kb) and 7 kb of upstream and downstream sequences, respectively⁷) or on a northern blot of mixed-stage poly(A)⁺

TABLE 1 *ced-9(+)* competes with the ability of *ced-9(n1950)* to prevent programmed cell death

Zygotic genotype	Maternal genotype	Extra cells in anterior pharynx	No. animals
+/+	+/+	0.03 ± 0.02*	60
<i>n1950</i> /+	+/+	5.3 ± 0.4*	25
<i>n1950/n1950</i>	<i>n1950/n1950</i>	13.3 ± 0.3*	45
<i>n1950</i> /+	<i>n1950/n1950</i>	10.5 ± 0.7	11
<i>n1950/Df</i>	<i>n1950/n1950</i>	13.6 ± 0.3	30
<i>n1950/ced-9(n1653)</i>	<i>ced-9(n1653)</i>	12.7 ± 0.3	21
<i>n1950</i>	<i>ced-9(n1950 n2161)</i>	13.6 ± 0.2	30
<i>ced-9(n1950 n2161)</i>			

Complete genotypes were, from top to bottom: +/+, wild-type (N2) progeny from wild-type parents; *n1950*/+, non-Unc progeny of *n1950* males crossed with *unc-69(e587)* hermaphrodites; *n1950/n1950*, *n1950* progeny from *n1950* parents; *n1950*/+, Unc-36 male cross-progeny of *eT1 unc-36/nDf40 dpy-18(e364)* males crossed with *unc-36(e251)* *n1950* hermaphrodites; *n1950/Df*, non-Unc progeny from the same cross; *n1950/n1950 n2161*, non-Unc progeny of *n1950* males crossed with *unc-69(e587)* *ced-9(n1950 n2161)* hermaphrodites; *n1950/n1653*, non-Unc progeny of *n1950* males crossed with *unc-69(e587)* *ced-9(n1653)* hermaphrodites. For comparative purposes, mutations that completely inhibit programmed cell death lead to the presence of 13 to 14 extra cells in the anterior pharynx (ref. 12 and our unpublished observations). Animals were anaesthetized with 30 mM NaCl (ref. 24) and observed using Nomarski optics microscopy²⁵. Cell survival was quantified as previously described¹². Nematodes were raised at 20 °C for all experiments described in this paper. Data shown are means ± standard errors on the mean (s.e.m.s.).

* Data from ref. 12. See ref. 12 for a description of the mutations used.

RNA (data not shown), suggesting that, unlike the *bcl-2*-activating mutations, *n1950* might be a point mutation. We therefore determined the nucleotide sequence of all *ced-9* exons in the *n1950* mutant. We found a single G-to-A transition, resulting in a substitution of glutamate (GAA) for glycine (GGA) at position 169 of the *ced-9* open reading frame. This G169E substitution affects an invariant residue located in a region highly conserved among all *ced-9/bcl-2* family members^{10,11} (Fig. 1a), suggesting that this site is important for the function of these proteins.

How might this point mutation activate *ced-9*? Perhaps *n1950* increases the activity or the stability of the CED-9 protein, possibly by inactivating a negative regulatory site. If so, then the

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death-suppressing activity of *n1950* might be enhanced by additional wild-type copies of *ced-9*. However, we observed the opposite effect: the addition of one wild-type copy of *ced-9* to animals carrying a single copy of the *n1950* allele decreased the ability of *n1950* to prevent programmed cell death (Table 1). Although *n1950*/deficiency animals had 13.6 extra surviving cells in the anterior pharynx, *n1950*/+ animals had only 10.5 extra cells ($P < 0.0001$). Animals *trans*-heterozygous for *n1950* and loss-of-function (*lf*) *ced-9* alleles (*n1653* and *n1950 n2161*) also showed higher levels of cell survival than did *n1950*/+ heterozygotes ($P < 0.005$ and $P < 0.0001$, respectively), indicating that this effect is not a result of the deletion of a linked gene by the deficiency. One possible interpretation of these results is that the CED-9(+) protein can antagonize the action of CED-9 (G169E).

To determine whether the G169E substitution we identified in *n1950* mutants was responsible for the *n1950* phenotype, we introduced this change into a 3.7 kb *NcoI*-*XhoI* genomic *ced-9*

fragment that rescues *ced-9(lf)* mutants from lethality⁷. We tested animals transgenic for this construct for the ability of the transgene to prevent programmed cell death in the presence or absence of wild-type CED-9 (provided by the endogenous locus). We found that animals transgenic for the G169E construct, but not for the wild-type gene, showed less cell survival in the presence of wild-type *ced-9* than in its absence (Fig. 1*b*). Thus, the G169E substitution can confer an *n1950*-specific characteristic to an otherwise wild-type *ced-9* gene, consistent with the hypothesis that this substitution causes the *n1950* phenotype.

To determine whether this point mutation might activate other members of the *ced-9/bcl-2* gene family, we introduced the corresponding G-to-E substitution (G145E) by site-directed mutagenesis of a human *bcl-2* cDNA, which we then put under the control of a *C. elegans* heat-shock (*hs*) promoter. *bcl-2* can prevent programmed cell death in *C. elegans* and can substitute for *ced-9* (refs 17, 18), suggesting that *ced-9* and *bcl-2* prevent cell

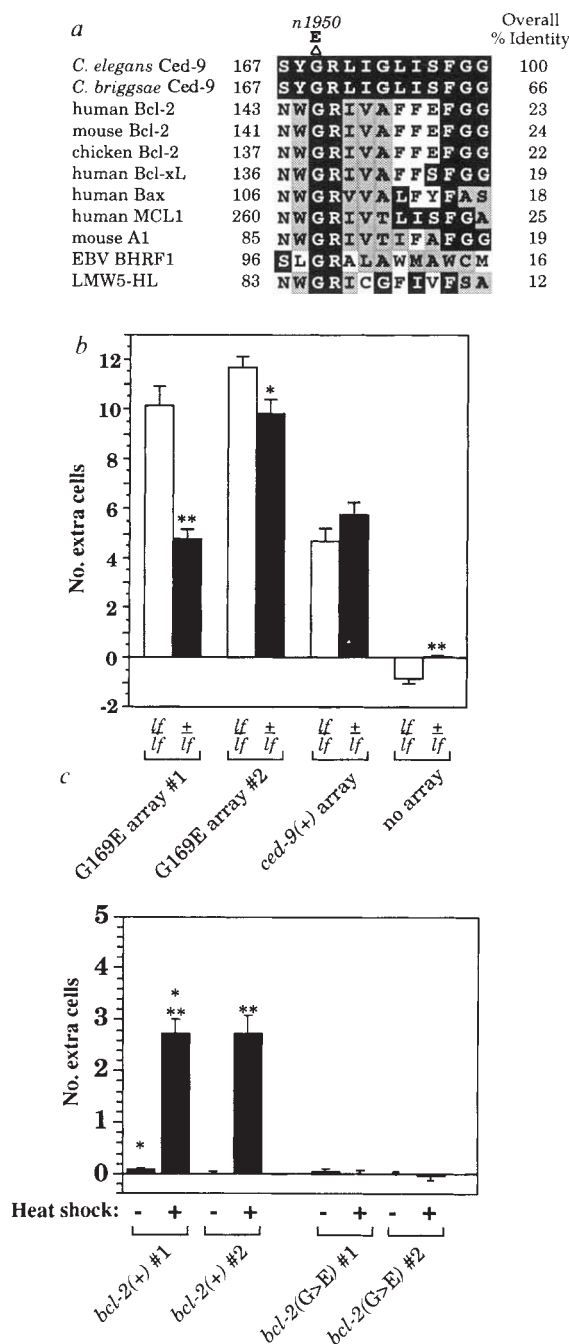


FIG. 1 The *n1950* mutation results in a G-to-E substitution in a region highly conserved among members of the *ced-9/bcl-2* gene family. **a**, Alignment of *C. elegans* CED-9 with *C. briggsae* CED-9 (ref. 7), human¹, mouse²⁶, and chicken²³ Bcl-2, human Bcl-x (ref. 4), human Bax (ref. 3), human MCL1 (ref. 5), mouse A1 (ref. 6), EBV BHRF1 (ref. 2), African swine fever virus LMW5-HL (ref. 27), and herpes virus saimiri ORF16 (ref. 9) in the conserved region around the *n1950* mutation. Residues identical to those of CED-9 are shown in white letters on black background; similar residues⁷ are shaded. Overall % Identity, per cent identity with *C. elegans* CED-9 over the entire protein. Residue numbers of the first residue shown for each protein are indicated. The change found in the *n1950* mutant is indicated above the line for *C. elegans*. **b**, The G169E substitution in an otherwise wild-type *ced-9* gene results in a protection against cell death that is antagonized by wild-type *ced-9*. No. extra cells, number of extra cells in the anterior pharynx of *ced-9(lf)/ced-9(lf)* (*ced-9(n1653)*) (empty bars) or *ced-9(+)/ced-9(lf)* (*qC1/unc-69(e587) ced-9(n1950 n2077)*) (filled bars) animals transgenic for a *ced-9(+)* or either of two independently isolated *ced-9*(G169E) arrays, or non-transgenic. The *ced-9*(G169E) arrays also caused more cell survival in *ced-9(n1950 n2077)* homozygotes than in *ced-9(n1950 n2077)/+heterozygotes* (data not shown). In the *ced-9(lf)* control, cells that normally survive undergo programmed cell death¹, resulting in fewer cells being present than in the wild type. * $P < 0.01$; ** $P < 0.0001$. At least 18 animals were scored for each genotype shown. **c**, Bcl-2(G145E) does not prevent programmed cell death in *C. elegans*. No. extra cells, number of extra cells in the anterior pharynx of animals transgenic for *hs-bcl-2(+)*¹⁸ or *hs-bcl-2*(G145) arrays. Animals were heat-shocked and scored as described¹⁸. Results for two independently isolated arrays are shown for each construct. Similar results were obtained with four additional arrays for each construct (data not shown). * Data from ref. 18. ** $P < 0.0001$. At least 20 animals were scored for each genotype shown.

METHODS. *ced-9* exons from wild-type and the *n1950* mutant were amplified by PCR and DNA sequences were determined as previously described². The single base pair change detected in *n1950* was introduced by site-directed mutagenesis into either a 3.7 kb *NcoI*-*XhoI* fragment (*ced-9(+)*) that rescues *ced-9(lf)* mutants from lethality¹² to create *ced-9*(G169E), or into a 1.9 kb human *bcl-2* cDNA¹⁶ (*bcl-2(+)*), to create *bcl-2*(G145E). Constructs were injected into animals of genotype *qC1/unc-69(e587) ced-9(n1950 n2077)*, using the plasmid pRF4 as a dominant co-injection marker²⁸. Genetic crosses were used to transfer established *ced-9* arrays into a *ced-9(n1653)* background. Cell survival was determined as in Table 1. Data shown are means $\pm$ s.e.m.s.

TABLE 2 *ced-9(lf)* mutations can increase the cell survival caused by weak *ced-3* mutations

<i>ced-3</i> Genotype	<i>ced-9</i> Genotype					
	<i>ced-9(+)</i>		<i>ced-9(n1653ts)</i>		<i>ced-9(n2812)</i>	
	Extra cells	<i>n</i>	Extra cells	<i>n</i>	Extra cells	<i>n</i>
Wild-type						
3+	0.03 ± 0.05	50	-0.84 ± 0.17**	50	-0.33 ± 0.14*†	30
Weak alleles						
3n2425	0.24 ± 0.08	29	0.84 ± 0.17*	37	ND	
3n2427	2.8 ± 0.3	25	8.6 ± 0.4**	30	7.7 ± 0.4**	25
3n2438	2.1 ± 0.2	50	8.0 ± 0.4**	30	8.4 ± 0.4**	30
Strong allele						
3n717	12.5 ± 0.4	30	ND		14.1 ± 0.3*	25

Numbers of extra cells in the anterior pharynx of weak *ced-3* mutants in wild-type or *ced-9(lf)* backgrounds. The weak *ced-3* alleles *n2425*, *n2427* and *n2438* were isolated as suppressors of the maternal-effect lethality caused by *ced-9(n1950 n2161)* (our unpublished results). The mutation *ced-9(n2812)* was isolated by S. Shaham (personal communication). The strong *ced-3* allele *n717* and the *ced-9* allele *n1653* have been described previously^{12,21}. In the *ced-9(lf)* control strains (genotype *ced-3(+)*), cells that normally survive undergo programmed cell death¹, resulting in fewer cells than in the wild type. Cell survival was determined as in Table 1. Data shown are means ± s.e.m. Different from *ced-9(+)* background at: * $P < 0.01$; ** $P < 0.0001$.

ND, not done.

† Animals of this genotype cannot be maintained as a strain; numbers shown are for homozygous animals derived from heterozygous mothers. Brood size determinations confirmed that the increase in cell survival observed in the *ced-9(lf)* background was not a result of the increased survival of animals with more extra cells (data not shown).

death by a common and conserved mechanism. We found that unlike *hs-bcl-2(+)* constructs, *hs-bcl-2(G145E)* constructs were unable to prevent programmed cell deaths in *C. elegans* (Fig. 1c), suggesting that this mutation might inactivate rather than activate Bcl-2. This mutation also eliminates Bcl-2 activity in mammalian cells¹⁹, apparently because it eliminates the ability of Bcl-2 to form heterodimers with Bax (a Bcl-2 homologue that stimulates rather than prevents apoptosis^{3,20}). Thus, despite their overall sequence and functional similarities, CED-9 and Bcl-2 are not completely equivalent. Perhaps there exists another mammalian member of the *ced-9/bcl-2* family that is functionally more similar to *ced-9* than is *bcl-2*. In addition, these observations suggest that the *n1950* mutation might result in altered affinity or specificity of CED-9 for other proteins.

How can we reconcile the findings that wild-type *ced-9* can prevent programmed cell death (Fig. 1b and ref. 7) and also can promote programmed cell death by antagonizing *n1950*. One possibility is that the CED-9(+) and CED-9(G169E) proteins compete for an essential cofactor, a protein activator, an addi-

tional subunit of a heteromeric complex that includes CED-9 or a protein target. In this way, CED-9(+) might lower the amount of factor available for CED-9(G169E), resulting in reduced protection from cell death. An alternative model is that wild-type CED-9 can exist in two forms, one that is present in living cells and protects from cell death and another that is present in dying cells and promotes cell death.

At least one known *ced-9* homologue, *bcl-x*, encodes both death-inhibiting and death-promoting forms⁴, providing a precedent for such a mechanism. The *n1950* mutation might render *ced-9* unable to promote cell death, while still allowing it to protect.

If CED-9(+) can promote the deaths of cells that are programmed to die, then such deaths might be reduced in animals that lack CED-9(+) activity. To test for such an effect, we sensitized cells that usually die to a change in *ced-9* activity. We constructed double mutants between *ced-9(lf)* mutations and weak alleles of the gene *ced-3*. Although null mutations in *ced-3* prevent all programmed cell deaths²¹, weaker alleles block cell death only partially (Table 2). If the sole function of *ced-9* is to prevent cell death, we would expect the same number of cells (or fewer) to survive if *ced-9* function is reduced. However, we found that *ced-9(lf); ced-3* double mutants had more surviving cells than did the corresponding *ced-3* single mutants; for example, the *ced-9(n1653); ced-3(n2438)* double mutant had about eight extra cells in the anterior pharynx, compared to about two extra cells in the *ced-3(n2438)* single mutant (Table 2). These findings support the hypothesis that *ced-9* function can promote cell death. That in the absence of *ced-9* function both cells that should live and cells that should die undergo programmed cell death¹² indicates that the death-promoting form of CED-9 is not essential for the process of programmed cell death but might help ensure that cells that should die do so reproducibly.

The *n1950* G169E substitution affects an invariant residue in a domain conserved among all *ced-9/bcl-2* family members. That a change at this position activates a member of this family strongly suggests that this region, and the glycine residue in particular, is involved in regulating the activity of these proteins. Could the equivalent substitution result in activation of other members of this family? Although the equivalent change appears to inactivate human Bcl-2 in both mammalian cells and *C. elegans* (ref. 19 and Fig. 1c), it remains possible that Bcl-2 activation might be seen for this mutant using different assays. Also, it is possible that only some family members are activated by such mutations. It is intriguing, for example, that the reported sequence of a chicken *bcl-2* complementary DNA isolated from a B-cell lymphoma library²² shows a valine instead of the glycine found in the genomic sequence²³ at this position (G139 in Fig. 1a). We speculate that this chicken lymphoma might, like certain lymphomas in humans, have been associated with an activation of *bcl-2*, caused in this case by the G139V mutation. □

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BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax

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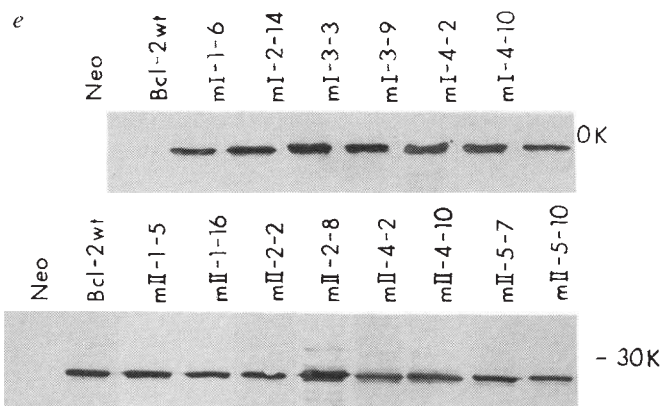
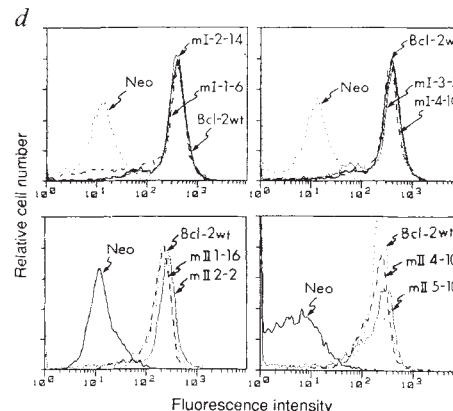
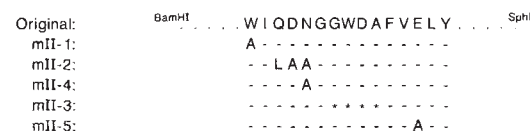
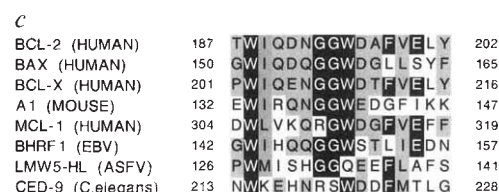
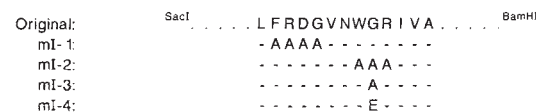
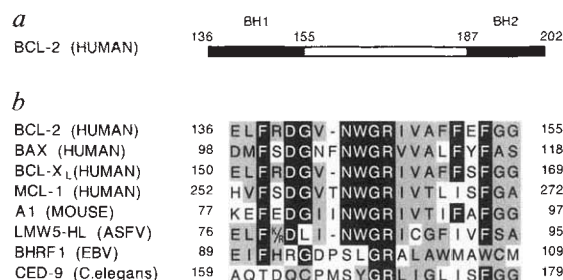
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BCL-2 was isolated from the t(14;18) chromosomal breakpoint in follicular B-cell lymphoma¹⁻³. Bcl-2 has the unique oncogenic role of extending cell survival by inhibiting a variety of apoptotic deaths⁴⁻¹³. An emerging family of Bcl-2-related proteins share two highly conserved regions¹⁴⁻²⁰ referred to here as Bcl-2 homology 1 and 2 (BH1 and BH2) domains (Fig. 1). This includes Bax which heterodimerizes with Bcl-2 and when overexpressed counteracts Bcl-2¹⁴. We report here that site-specific mutagenesis of Bcl-2 establishes the two domains as novel dimerization motifs. Substitution of Gly 145 in BH1 domain or Trp 188 in BH2 domain completely abrogated Bcl-2's death-repressor activity in interleukin-3 deprivation, γ -irradiation and glucocorticoid-induced apoptosis. Mutations that affected Bcl-2's function also disrupted its heterodimerization with Bax, yet still permitted Bcl-2 homodimerization. These results establish a functional role for the BH1 and BH2 domains and suggest Bcl-2 exerts its action through heterodimerization with Bax.

The most highly conserved domains in the Bcl-2 family, BH1 and BH2 are separated by about 30 amino acids (Fig. 1a-c). Mutagenesis of the most conserved amino acids in both domains was undertaken (Fig. 1b, c). Mutant Bcl-2 proteins were expressed in FL5.12 and 2B4 cell lines^{7,21}. Clones stably expressing comparable levels of Bcl-2 mutant (mI, mII) or wild-type (wt) protein were selected by flow cytometry (Fig. 1d) and western blot analysis (Fig 1e).

FIG. 1 Stable clones expressing mutant Bcl-2. **a**, Schematic representation of the BH1 and BH2 domains. **b** and **c**, Sequence comparison of BH1 and BH2 domains among Bcl-2 family members, respectively¹⁴⁻²⁰. The alignment was maximized by introducing insertions marked by dashes. Identical amino acids are in black, conserved residues are shaded. Under each domain a schematic representation of the amino-acid substitutions in each mutant (mI or mII) uses dashes to indicate no change and stars to indicate a deletion. **d**, Expression of wild-type (wt) and mutant (mI, mII) Bcl-2 in representative FL5.12 clones, detected by flow cytometry. Neo, clone transfected with vector lacking insert. The last digit of the mutants indicates the clone number. **e**, Western blot of representative stable FL5.12 clones developed with anti-human 6C8 Bcl-2 antibody. 2B4 clones with comparable Bcl-2 levels were also selected (not shown).

METHODS. Bcl-2 mutants were generated by PCR-mediated site-directed mutagenesis²⁹. An EcoRI fragment of human Bcl-2 cDNA³⁰ was cloned into a modified pBluescript II KS vector (Stratagene), whose SacI and BamHI sites had been eliminated. A 153-bp SacI-BamHI fragment containing the BH1 domain was replaced with a PCR-synthesized fragment containing substituted nucleotides. For mII-2, 3, 4, 5, a 60-bp BamHI-SphI fragment containing the BH2 domain was replaced with a fragment containing substituted nucleotides by means of mutually primed synthesis²⁹. For mI-1, because Trp 188 is at the BamHI site, a 153 bp SacI-BamHI fragment was replaced with a synthesized fragment containing Ala 188. All constructs were sequenced to ensure that only specified positions were modified. The mutated Bcl-2 molecules were cloned into the pSFV-Neo vector and transfected as previously described^{6,14}. Clones were picked at random and examined for expression of human Bcl-2. Flow cytometry and western blots were conducted as described^{6,14,27}.



FL5.12 clones were assessed in a cell-death assay following deprivation of interleukin-3 (IL-3). Within the BH1 domain, alanine replacement of FRDG (mI-1) or WGR (mI-2) markedly decreased the death-repressor activity of Bcl-2 in each of 3 clones tested (Fig. 2a). Clones bearing mutant proteins revealed a morphological apoptotic death with oligosomal-length DNA fragmentation and DNA release (Fig. 2b)²².

The frequent structural importance of glycine residues²³ prompted the substitution of Gly 145 with alanine (mI-3). All five mI-3 clones tested lacked death-repressor activity (Fig. 2a, b). This same glycine position was changed to glutamate in the gain-of-function mutation in *ced-9* (ref. 24). CED-9 is the Bcl-2 homologue in *Caenorhabditis elegans*²⁵. Consequently, the same glutamic acid substitution was created in Bcl-2 (mI-4), but proved to be loss-of-function when assessed in mammalian cells (Fig. 2a, b). To assess whether these subtle modifications would eliminate Bcl-2 activity in other death pathways, constructs were introduced into 2B4 cells. Neither mI-3 or mI-4 Bcl-2 proteins could block glucocorticoid-induced (Fig. 2c) or γ -irradiation-

induced cell death (data not shown). All clones of each mutant died with similar kinetics. The clones bearing mI-4 mutants displayed increased death compared to control cells (Neo) (Fig. 2).

Within the BH2 domain the Trp residue (Trp 188 in Bcl-2) is universally present in all family members (Fig. 1c). Replacement of this amino acid with Ala (mII-1) abrogated Bcl-2's inhibition of apoptosis (Fig. 2d). The QDN 190–192 motif and Glu 200 are conserved in many family members. Clones expressing Bcl-2 with substitutions at these positions (mII-2 and mII-5, respectively) displayed about half the death-repressor activity of wild-type Bcl-2. However, a substitution of Ala for Asn 192 (mII-4) had no effect on Bcl-2's death-repressor activity (Fig. 2d).

A Bcl-2 mutant (mII-3) that deleted the conserved GWDA 194–197 motif also completely eliminated Bcl-2's ability to block apoptosis. However, the level of mII-3 protein was lower than that of Bcl-2 wild type, suggesting that this mutation also affected protein stability (not shown). The mII-1 Bcl-2 protein also failed to protect 2B4 cells from dexamethasone- or γ -irradiation-induced death, whereas the mII-2 protein again displayed about half the activity (data not shown).

Because Bax counters Bcl-2 activity and forms heterodimers with Bcl-2 (ref. 14), we examined each Bcl-2 mutant for association with Bax. Immunoprecipitations revealed that all mutants that eliminated Bcl-2's death-repressor activity failed to interact with Bax. This includes the single substitutions of Gly 145 or Trp 188 (Fig. 3a, b). The mII-3 Bcl-2 protein, although present in lower amounts, also failed to associate with Bax (not shown). The mII-2 and mII-5 proteins, which displayed decreased death repression, also demonstrated a diminished interaction with Bax. In contrast, mII-4 protein, which fully protected cells from death, interacted with Bax to an extent similar to Bcl-2 wild type (Fig. 3b). Bcl-2 mutants expressed in 2B4 cells showed the same pattern of heterodimerization (not shown). Thus, the same amino acids required for Bcl-2 function were also needed for heterodimerization with Bax.

To determine whether Bcl-2 could form homodimers and if the same mutations would affect its homodimerization, three approaches were taken. First, crosslinking with DSP²⁶ revealed 25K monomeric Bcl-2 as well as complexes at 46K and 50K, the predicted size of Bcl-2/Bax heterodimers and Bcl-2 homodimers, respectively (Fig. 4a). Second, wild-type human Bcl-2 bearing

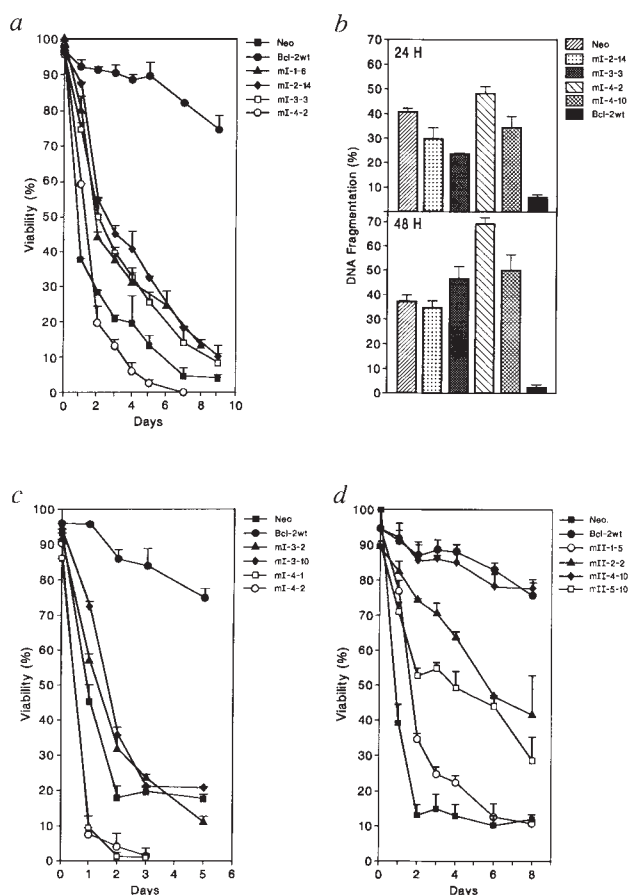


FIG. 2 Cell death assays of stably transfected clones. a, Viability after IL-3 deprivation of representative FL5.12 clones bearing Neo control, Bcl-2 wt, or BH1 mutant (mI) vectors. b, Percentage of DNA fragmentation in representative FL5.12 clones following IL-3 deprivation for 24 or 48 h. c, Viability assay of representative 2B4 clones bearing Neo control, Bcl-2 wt, or BH2 mutant (mII) vectors after IL-3 deprivation. All data shown are mean $\pm$ s.d. from triplicate samples.

METHODS. Apoptosis was induced in FL5.12 cells by IL-3 deprivation as described⁷. For glucocorticoid-induced apoptosis, 6.8×10^4 2B4 cells were cultured with 10^{-6} M dexamethasone in 96-well plates. Viability was determined at designated time points by trypan blue exclusion. All experiments were done at least three times with at least 3–6 individual clones for each mutation. Quantification of DNA fragmentation in FL5.12 cells following IL-3 deprivation was measured by the diphenylamine method⁸.

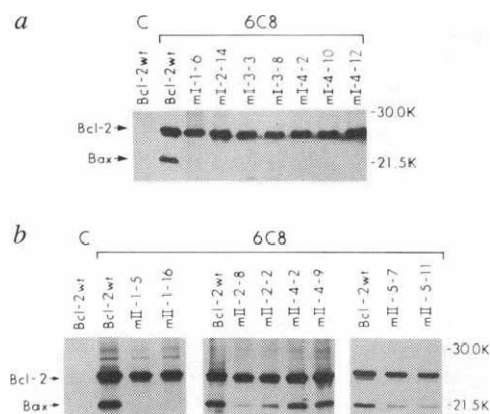


FIG. 3 Immunoprecipitation of Bcl-2 wild-type and mutant proteins. a and b, FL5.12 clones expressing wild-type or BH1 (a) or BH2 (b) mutant Bcl-2 were either immunoprecipitated with a control hamster antibody (C) or the 6C8 antibody.

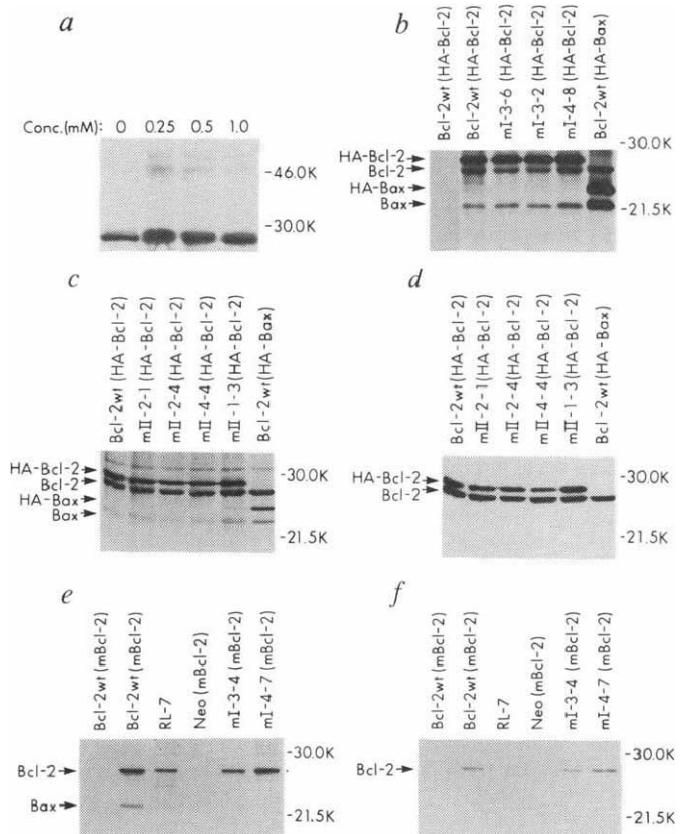
METHODS. Immunoprecipitations were done as described¹⁴. Briefly, equal amounts of cells per sample ($5-10 \times 10^6$ cells) were labelled overnight with 100 μ Ci of 35 S-Met and lysed in 0.25% NP-40 buffer. Immunoprecipitation was conducted using the 6C8 antibody followed by protein A-Sepharose beads. Proteins were separated by electrophoresis on a 12.5% SDS-polyacrylamide gel, fixed with 10% glacial acetic acid and 30% methanol, and treated with a fluorography enhancing solution (EN³HANCE, DuPont)¹⁴.

FIG. 4 Analysis of wild-type and mutant Bcl-2 homodimers. **a**, Western blot developed with 6C8 anti-Bcl-2 antibody following crosslinking of postnuclear supernatants of FL5.12-hu Bcl-2 wt lysed in 0.25% NP-40 buffer with DSP (dithiobis (succinimidyl propionate)) at the indicated concentrations²⁶. **b**, Immunoprecipitation of radiolabelled doubly transfected clones with either control antibody (lane 1) or 12CA5 anti-HA antibody¹⁴ (lanes 2–6). FL5.12 clones that possessed either wt or mutant (ml) human Bcl-2 were subsequently transfected with HA-tagged human Bcl-2 wt or HA-Bax as indicated in parenthesis. **c**, Immunoprecipitation of FL5.12 cells doubly transfected with Bcl-2 wt or BH2 mutants (mII), and subsequently HA-Bcl-2 or HA-Bax (as indicated in parenthesis). Radiolabelled cells were immunoprecipitated with anti-HA antibody. After SDS-PAGE gel separation and transfer to nitrocellulose paper, the blot was dried and exposed for ³⁵S-Met signals. **d**, The same blot shown in **c** was stained with biotinylated anti-human Bcl-2 antibody and developed with ECL substrate. **e**, Immunoprecipitation of ³⁵S-Met radiolabelled, doubly transfected FL5.12 clones or RL-7, a human B-cell line expressing high levels of Bcl-2¹⁴, with either control antibody (lane 1) or 6C8 antibody (lanes 2–6). Clones were first transfected with wt or mutant human Bcl-2 and secondarily re-transfected with mouse Bcl-2 wt as indicated in parenthesis. **f**, Western blot from the same immunoprecipitation shown in **e** developed with biotinylated 3F11 anti-mouse Bcl-2 antibody.

METHODS. The HA-Bcl-2 construct was human Bcl-2 wt tagged with a haemagglutinin epitope¹⁴ and cloned into an RSV promoter-driven expression vector. For secondary transfections constructs were cotransfected with the LAP267 vector¹⁴ and selected with 2 mg ml⁻¹ hygromycin. The short period of exposure used for ECL (Amersham) development was not sufficient for a ³⁵S signal to be detected (**d** and **f**). After western blot results were obtained, the blot was washed with PBS containing 0.05% Tween-20 to strip the ECL substrate and exposed at room temperature to reveal immunoprecipitated ³⁵S-Met-labelled proteins (**c** and **e**).

an epitope of the influenza virus haemagglutinin¹⁴ (HA-Bcl-2) was introduced into FL5.12 cells. The HA-specific antibody (12CA5) coprecipitated wild-type Bcl-2 and Bax along with the HA-Bcl-2 molecule, indicating the presence of Bcl-2 homodimers (Fig. 4b, c). Moreover, BH1 mutants (mI-3 and mI-4) and BH2 mutants (mII-1 and mII-2) still demonstrated association with HA-Bcl-2. Western blots of these immunoprecipitates confirmed that equivalent amounts of human origin Bcl-2 wild type or mutant protein were coprecipitated (Fig. 4d). Finally, murine Bcl-2 was overexpressed in FL5.12 cells possessing either human Bcl-2 wild type or mutant proteins (Fig. 4e, f). Immunoprecipitations and subsequent western blots using species specific antibodies²⁷ revealed that mutant as well as wild-type human Bcl-2 proteins dimerized with mouse Bcl-2 (Fig. 4f). Taken together, these results indicate that Bcl-2 is able to form homodimers and this property is left intact by the selected mutations which abrogated function and heterodimerization.

Both BH1 and BH2 domains proved critical for Bcl-2's function and the formation of Bcl-2/Bax heterodimers. Select mutations in BH1 or BH2 domains still enabled Bcl-2 to homodimerize, yet the Bcl-2 homodimers were insufficient to protect cells from death. We cannot exclude that conserved Bcl-2 residues such as Gly 145 or Trp 188 might have additional roles, but each amino acid is clearly required for heterodimerization with Bax. In addition, Bcl-2 appears to have further partners²⁸, and could prove to have additional functional domains. However, the correlation between the ability of Bcl-2 to repress cell death and its ability to heterodimerize with Bax is striking. The conservation of the novel BH1 and BH2 dimerization motifs in an expanding Bcl-2 family (Fig. 1) suggests other members may also participate in competing dimerizations. The same BH1 glycine residue is altered in a CED-9 gain-of-function mutation, emphasizing the importance of this protein interface in the regulation of a cell death pathway that appears to be universal to multicellular organisms. Overall, the current data favour a model in which Bcl-2 must bind Bax to exert its death-repressor activity



and suggest that strategies that disrupt Bcl-2/Bax heterodimers would be expected to promote cell death. □

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Binary and ternary complexes between T-cell receptor, class II MHC and superantigen *in vitro*

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SUPERANTIGENS are proteins that in association with class II major histocompatibility complex (MHC)-bearing cells can stimulate virtually all T cells that express particular classes of the variable β -domains of the T-cell receptor (TCR)¹. This mechanism of T-cell activation circumvents the usual requirement for peptide-specific MHC recognition. *Staphylococcus aureus* enterotoxin B (SEB) is a bacterial superantigen that causes food poisoning and shock²⁻⁵. We have characterized the tertiary complex of SEB, a soluble T-cell receptor, and a soluble class II MHC molecule DR1, and the three binary complexes TCR-SEB, SEB-DR1, and the peptide-specific complex DR1-TCR. We report here that in each case the specificity of the interaction among the soluble molecules is the same as observed in biological assays. Native gel electrophoresis and plasmon resonance affinity measurements indicate that SEB-TCR complex can form in the absence of class II MHC and that SEB-TCR interaction increases the binding of DR1. The observation that a superantigen can form complexes with TCR in both the absence and presence of class II MHC may provide a mechanism for its ability to induce anergy in some circumstances and activation in others^{6,7} (reviewed in ref. 8).

To investigate the interactions of TCR, class II MHC and SEB, we used a soluble human TCR $\alpha\beta$ heterodimer (TCR HA1.7) which has specificity for influenza haemagglutinin HA peptide (306-318) bound to the human class II MHC molecule HLA-DR1⁶. The variable (V) gene segment of the β -chain of TCR (HA1.7) is V β 3.1, which has specificity for SEB⁹. A native gel-shift assay (Fig. 1) was used to examine formation of complexes between TCR, DR1 and SEB under non-denaturing conditions. TCR (lane 1), SEB (lane 4), and DR1-HA (HA peptide 306-318 bound to HLA-DR1¹⁰) (lane 6), migrate through a native polyacrylamide gel with distinct mobilities. Co-incubation of TCR, DR1-HA and SEB resulted in a new band (lane 2,***), indicating formation of a complex. The presence of TCR, DR1 and SEB in this band was confirmed by immunoblot analysis using antibodies specific for each protein (Fig. 1b). These data confirm the existence of the predicted ternary complex⁵.

No complexes are observed under these conditions between DR1 and SEB in the absence of TCR (Fig. 1a, lane 3) or between DR1-HA and TCR in the absence of SEB (Fig. 1a, lane 5). This could result from weaker affinities and/or fast dissociation rates of these two binary complexes, which are beyond the sensitivity of detection in native gels. Affinity of the TCR/MHC class II

interactions has been reported to be low (dissociation constant $K_D \sim 10^{-4}$ to 10^{-5} M)¹¹⁻¹³.

The binary complex between SEB and TCR (in the absence of DR1) is detected as a band on native gel electrophoresis (Fig. 2a, lane 6,***) with mobility distinct from that of SEB (lane 7) or TCR (lane 8).

The specificity of the interactions among soluble TCR, superantigen and class II MHC molecules was also studied with native gel electrophoresis. The ternary complex TCR-SEB-DR1 was observed with four different peptides bound to DR1 (Fig. 2a; compare lanes 1-4 to lanes 10-13). This indicates that *in vitro*, as *in vivo*, superantigen circumvents the usual requirement for peptide-specific MHC recognition by TCR. The slight variation in mobility of the ternary complex bands (lanes 1-4,****) reflects differences in the mobilities of HLA-DR1 bound to different peptides (lanes 10-13). This observation, as well as the disappearance of the DR1 and TCR bands in lanes 1-4 (Fig. 2a), provides additional evidence for the presence of all three protein molecules in the ternary complex band.

The specificity of this TCR (HA1.7, V β 3.1) for the superantigen SEB¹⁴ but not SEA (*S. aureus* toxin A) or TSST-1 (toxic-shock-syndrome toxin 1)¹⁵ was observed with native gel electrophoresis. SEB formed a complex with TCR in both the absence (Fig. 2a, lane 6; Fig. 2b, lane 3) and presence (Fig. 2a, lane 4,*** and Fig. 2b, lane 4) of DR1, whereas TSST-1 (Fig. 2b, lanes 5, 6) and SEA (not shown) show no complex formation with this TCR. Immunoblot analysis with a TCR antibody (Fig. 2c) confirmed that this TCR did not form a complex with TSST-1 in the absence (lane 2) or presence (lane 3) of DR1.

We have also observed the ternary and binary complexes using a BIAcore instrument^{16,17} that can detect the binding of one or more soluble proteins to a protein immobilized on a dextran-coated chip as a difference in refractive index¹⁸. Figure 3 shows the binding of proteins to immobilized TCR. SEA (1 μ M) shows no significant binding, whereas SEB (1 μ M) clearly forms a complex with TCR (Fig. 3). DR1-HA at high concentration (25 μ M) shows a small binding signal, whereas DR1-HA (1 μ M) pre-mixed with SEB (1 μ M) binds to the TCR surface with a large signal (much greater than additive), indicating formation of the ternary complex (Fig. 3a). In control experiments, neither SEB nor DR1-HA bound significantly to the dextran matrix alone; and other proteins for which the TCR is not specific, such as DR1-I24 or HLA-A2 (class I MHC), did not bind to the immobilized TCR (Fig. 3b, and data not shown). Although high DR1-HA concentration (25 μ M; Fig. 3) was required to observe binding to immobilized TCR, the binding was peptide-specific. DR1 complexed with another peptide (I24) showed no binding at 25 μ M (Fig. 3b). On the basis of experiments using DR1-HA concentrations up to 50 μ M, we estimate that the K_D of the DR1-HA, TCR (HA1.7) interaction is greater than 25 μ M *in vitro*.

With SEB immobilized on a chip, both the binary complexes DR1-SEB and TCR-SEB were readily detected (Fig. 4A, B). In control experiments, neither DR1 (Fig. 4Aa, curve 2) nor TCR (not shown) bound to the free dextran surface; and HLA-A2 (class I MHC), which does not have specificity for SEB, did not bind to the immobilized SEB (not shown). In Fig. 4A a binary complex between DR1 and SEB is observed, which was not detectable by native gel electrophoresis (see above). Figure 4B provides further evidence that SEB can form a stable binary complex with TCR in the absence of MHC class II.

Equilibrium dissociation constants and kinetic on- and off-rates were measured for the novel TCR-SEB complex. Kinetic constants were calculated from the ascending rate of the BIAcore signal during binding and the descending rate during wash-off (Fig. 4, legend). For TCR binding to immobilized SEB, an on-rate constant ($k_{on} = 1.30 \pm 0.12 \times 10^4$ M⁻¹ s⁻¹) and off-rate constant ($k_{off} = 1.06 \pm 0.05 \times 10^{-2}$ s⁻¹) were determined from triplicate experiments (Fig. 4B). From their ratio, the equilibrium dissociation constant, $K_D = 0.82$ μ M, is estimated for the TCR-

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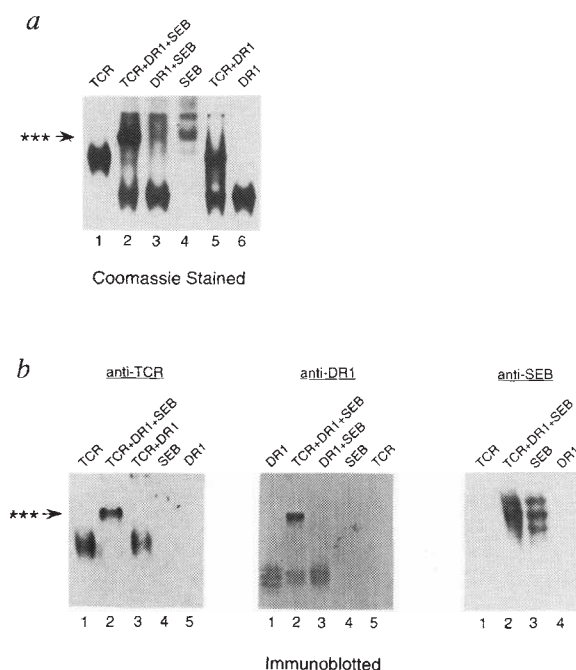


FIG. 1 Native gel shift assay. *a*, Co-incubation of DR1-HA (HLA-DR1 bound to HA 306–318 peptide), SEB and TCR (lane 2), results in the appearance of a distinct band (***), not observed with TCR (lane 1), SEB (lane 4) or DR1-HA (lane 6) alone, indicating TCR–SEB–DR1 complex formation. Co-incubation of DR1-HA and SEB (lane 3) or of DR1-HA with TCR (lane 5) did not result in any distinct mobility shift. *b*, Anti-TCR blot (α F1 mAb; T Cell Sciences) shows that TCR is shifted by incubation with DR1-HA and SEB; anti-DR1 blot (L243 mAb American Type Culture Collection II HB55) shows that DR1-HA is shifted by incubation with TCR and SEB; anti-SEB blot (polyclonal serum; Toxin Technology) shows that SEB is shifted by incubation with TCR and DR1-HA. Thus all three proteins are present in the band (***).

METHODS. HLA-DR1 (DRB1*0101), was produced in insect cells as a soluble protein, purified and complexed *in vitro* with peptides as published¹⁰. A soluble human $\alpha\beta$ T-cell receptor (HA1.7, V β 3.1 V α 1.2) was expressed and isolated as previously described for a mouse TCR²¹ (I.E. and T.O., unpublished results). SEB was purchased from Sigma. The TCR, peptide-complexed DR1 and SEB used in these experiments appear homogeneous by gel-filtration HPLC and by SDS–PAGE. The multiple SEB bands in native-PAGE may result from charge heterogeneity. Non-denaturing native-PAGE was done by omitting SDS in a PAGE system²². Proteins (15 μ M TCR, 20 μ M DR1, 35 μ M SEB) were incubated alone, or in combination, in PBS, with 1 mM phenylmethylsulphonyl fluoride, 10 μ g ml⁻¹ leupeptin, pH 7.0, at 37 °C for 18 h before non-reducing native polyacrylamide (10%) gel electrophoresis. No reducing agents were added and samples were not heated. The resolving gel buffer was 0.375 M Tris, pH 8.8. In native-PAGE the direction of migration of the proteins will depend on the buffer pH relative to the isoelectric point (pI) of the protein²³. The gels were run at 15 V cm⁻¹. Protein bands were visualized with *a*, Coomassie brilliant blue R250, or *b*, were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore), probed with specific antibodies, and the immune complexes visualized by alkaline phosphatase²⁴.

SEB complex. A similar value ($K_D=0.68 \mu$ M) was obtained from a Scatchard analysis (not shown) of equilibrium binding at various TCR concentrations (Fig. 4*Ba*). The equilibrium dissociation constant for the DR1–SEB complex was also measured. Scatchard analysis (Fig. 4*Ac*) of the data in Fig. 4*Ab* gave a $K_D=1.7 \mu$ M, a value similar to that measured for SEB binding to DR1 on cell surfaces¹⁹.

The kinetics of DR1–SEB association and dissociation were

too fast to be measured accurately in the experiments shown in Fig. 4*A*. The fast dissociation kinetics may be the reason that the DR1–SEB complex was not detectable in the native gel-shift assay (see above).

Our native gel electrophoresis results indicate that DR1 with peptide binds better to the SEB–TCR complex than to either molecule separately. TCR SEB–DR1 complexes could be observed for four different DR1–peptide complexes under condi-

FIG. 2 Specificity of TCR–SEB and TCR–SEB–DR1 complex formation. *a*, In the presence of SEB, TCR migrates with a mobility (lane 6, **) intermediate between SEB (lane 7) or TCR (lane 8) alone. Formation of the TCR–SEB–DR1 complex (lanes 1–4, ***) was independent of the peptide bound to DR1 (peptide sequences listed below), and was accompanied by a band shift relative to peptide-bound DR1 (lanes 10–13) and TCR alone (lane 8). *b*, The interaction of TCR with SEB was compared to the interaction of TCR with TSST-1. No complexes were detected when TCR was incubated with TSST-1 (lanes 5 and 6) or SEA (data not shown), but TCR–SEB (lane 3) and TCR–SEB–DR1 (lane 4, ***) complexes were observed. Neither TSST-1 nor SEA are reported to interact with V β 3.1 (ref. 1). *c*, Anti-TCR immunoblot of native gel with α F1 mAb specifically confirms that TCR is not shifted by incubation with TSST-1 (lane 2) or TSST-1+DR1 (lane 3), but is shifted by incubation with SEB and DR1 (lane 4).

METHODS. Proteins (10 μ M each) were incubated, subjected to native-PAGE (8%), and visualized as in Fig. 1. *a* and *b*, Coomassie stained. *c*, Anti-TCR immunoblot. Peptides: HA, influenza haemagglutinin (306–318) PKYVKQNTLKLAT; I24, class II-associated invariant chain (96–119), LPKPPKPVSKMRMATPLLMQALPM; I15, class II-associated invariant chain (105–119), KMRMATPLLMQALPM; MA, influenza matrix (17–29), SGPLKAEFAQRLE.

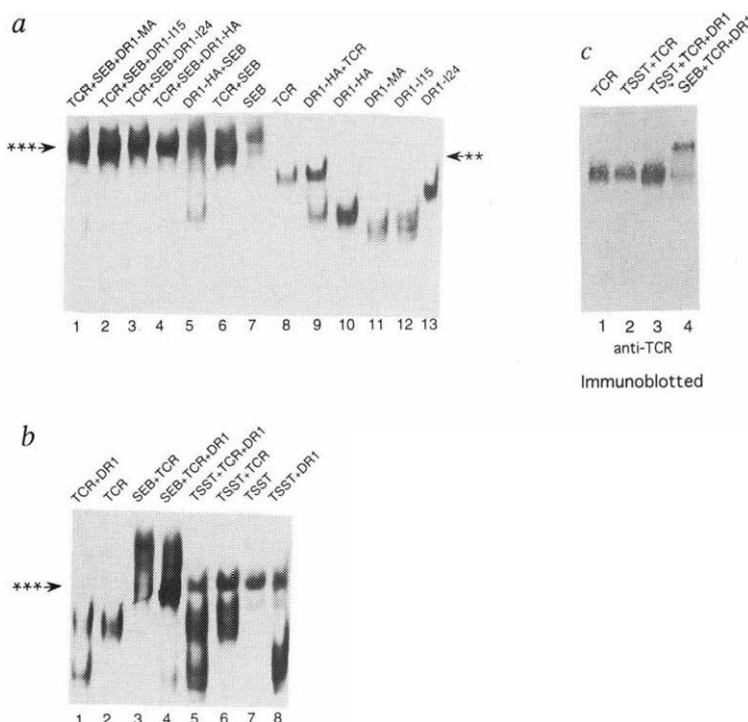
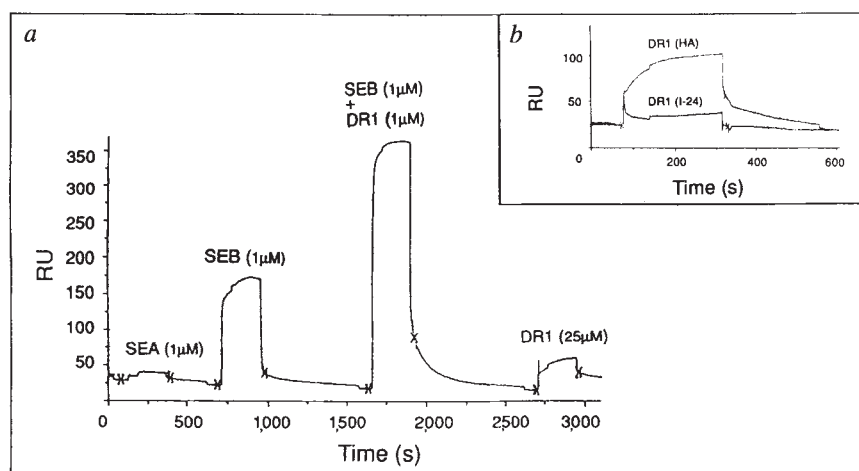


FIG. 3 Interaction of SEB, SEA, DR1 and pre-mixed (DR1+SEB) to immobilized TCR. Protein-protein interactions detected by surface plasmon resonance (BIAcore instrument, Pharmacia), where increase in RU (resonance units) indicates binding of injected protein to protein immobilized on surface. *a*, SEB (1 μ M) but not SEA (1 μ M) binds to immobilized TCR. Although DR1-HA binds only weakly even at concentrations as high as 25 μ M, premixing of SEB plus DR1-HA at equimolar (1 μ M) concentrations before injection resulted in a more than additive increase in binding signal. *b*, Overlaid sensorgrams comparing binding of immobilized TCR to DR1 bound to the correct antigenic peptide (DR1-HA, 25 μ M) versus DR1 bound to the control peptide (DR1-I24, 25 μ M).

METHODS. TCR was coupled to the dextran matrix by standard amine chemistry²⁵. A flow of HBS (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.5% surfactant P20 pH 7.4) was maintained over the sensor surface at 5 μ l min⁻¹. Samples were injected at 5 μ l min⁻¹ for 5 min each. Between injections, the surface was regenerated with HBS. The immobilization level was 3,000–4,000 RU. 'X', Resonance signal before the injection, at the height of the response



during the injection and towards the end of the wash. Although binding of DR1 and SEB to the immobilized TCR is detectable, baseline drift made kinetic and affinity measurements impractical.

tions where TCR-SEB complex formed, but DR1-SEB complexes were not stable (Figs 1 and 2). This suggests that either soluble $\alpha\beta$ TCR alters the conformation of SEB to a form which has higher affinity for DR1, or that $\alpha\beta$ TCR directly contacts parts of the DR1 molecule in the TCR-SEB-DR1 complex. Such a direct contact has previously been suggested based on variations in T-cell responses with MHC class II polymorphism, with the conformational state of MHC class II, and with the

type of TCR α chain (for review see ref. 20); this contact could occur on a DR1 surface adjacent to the bound peptide.

Two observations reported here indicate that TCR-SEB-DR1 complexes can be formed from soluble molecules. First, native gel electrophoresis of mixtures of the three molecules reveals a band with mobility distinct from the three individual constituents, or their binary complexes, and that band has been shown by immunoblotting to contain all three molecules (Figs 1 and

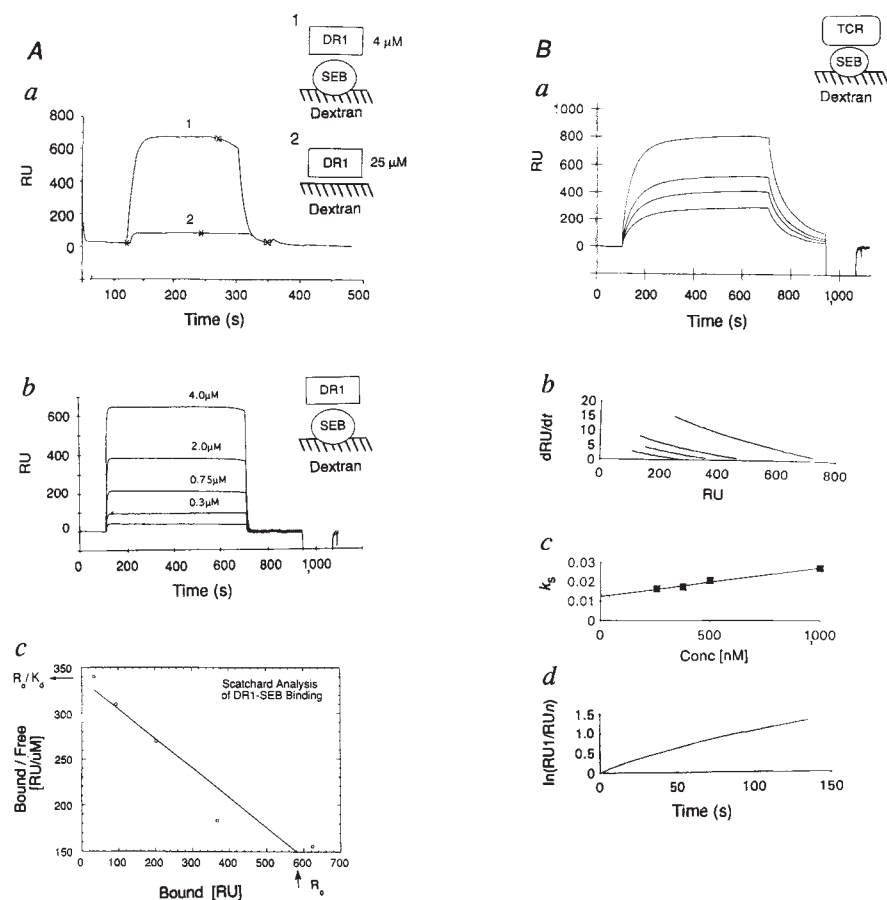


FIG. 4 Measurement of binding parameters for DR1-SEB (A) and TCR-SEB (B) complexes. *Aa*, DR1 binding to SEB surface (trace 1) versus control surface (trace 2); *b*, Concentration-dependent binding of DR1 to SEB surface; *c*, Scatchard plot intercepts give R_0 (maximum DR1-HA bound) and R_0/K_D , yielding a K_D of 1.7 μ M. *Ba*, Bottom to top traces, TCR $\sim$ 0.25 μ M, 0.35 μ M, 0.5 μ M, 1.0 μ M, binding to SEB. *b*, Binding rates dRU/dt at these TCR concentrations versus relative response in resonance units, RU. *c*, Association rates (k_s) versus TCR concentration (k_s = slopes from *b*). An apparent association rate constant ($k_{on} = 1.30 \pm 0.12 \times 10^4$ M⁻¹ s⁻¹; $n=3$) was calculated for TCR binding to SEB. *d*, Dissociation of bound TCR (1 μ M trace) from immobilized SEB. Assuming a single exponential decay, $k_{off} = 1.06 \pm 0.05 \times 10^{-2}$ s⁻¹ ($n=3$). The ratio of off- and on-rate constants gives an apparent equilibrium dissociation constant $K_D = 0.8$ μ M. The data shown here are from a typical experiment. The values for kinetic constants are mean $\pm$ s.d. from n independent experiments. Methods as in Fig. 3.

2). Second, plasmon resonance measurements of SEB and DR1 binding to an immobilized TCR indicate that much more DR1 binds to the immobilized TCR in the presence of SEB than binds under the same conditions in the absence of SEB (Fig. 3). We also confirm the weak affinity for MHC class II with peptide to TCR^{11,13}, demonstrating here that both soluble proteins interact in a peptide-specific manner as required for MHC restriction. Finally, we have observed direct binding of SEB to $\alpha\beta$ TCR in the absence of a class II MHC molecule ($K_D = 0.82 \mu\text{M}$) which exhibits kinetics ($k_{\text{on}} = 1.30 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$; $k_{\text{off}} = 1.06 \times 10^{-2} \text{ s}^{-1}$)

that suggest a half-life of the complex of 90 s. Although several studies report that activation of T cells by superantigen requires the presence of MHC class II molecules, under certain circumstances (crosslinking of toxin and/or presence of co-stimulant) superantigen can activate T cells in the absence of class II molecules (reviewed in refs 1, 8, 15). In the absence of co-stimulants or crosslinking, SEB alone appears to cause anergy⁶. The finding that superantigen complexes with TCR in both the presence and the absence of class II MHC *in vitro* may correlate with these different activities *in vivo*. □

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Binding of phosphatidylinositol-3-OH kinase to CD28 is required for T-cell signalling

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THE engagement of CD28 with its ligand B7.1/CD80 results in potent costimulation of T-cell activation initiated through the CD3/T-cell receptor complex^{1,2}. The biochemical basis of CD28 costimulatory function is poorly understood. The signalling pathways used by CD28 are unlike those used by the CD3/T-cell receptor in that they are resistant to cyclosporin A and independent of changes in cyclic AMP concentrations³. These differences suggest that each pathway provides unique biochemical information which is required for T-cell activation. We report here that CD28 becomes tyrosine-phosphorylated following interaction with B7.1/CD80, which induces formation of a complex with phosphatidylinositol-3-OH kinase, mediated by the SH2 domains of the p85 subunit of the kinase. Phosphatidylinositol-3-OH kinase is a heterodimer of this 85K regulatory subunit and a 110K catalytic subunit, and is a common substrate for most receptor tyrosine kinases and some cytokine receptors^{4,5}, binding through its SH2 domain to phosphotyrosine in the motif Tyr-X-X-Met in the CD28 sequence, which is highly conserved between human, mouse and rat^{6–8} and lies in the intracellular domain. We show that CD28 mutants that have their kinase-binding site deleted or the tyrosine at position 173 substituted by phenylalanine do not associate with the kinase after CD28 stimulation and cannot stimulate production of interleukin-2. Our results suggest that phosphatidylinositol-3-OH kinase is critical for signalling by CD28.

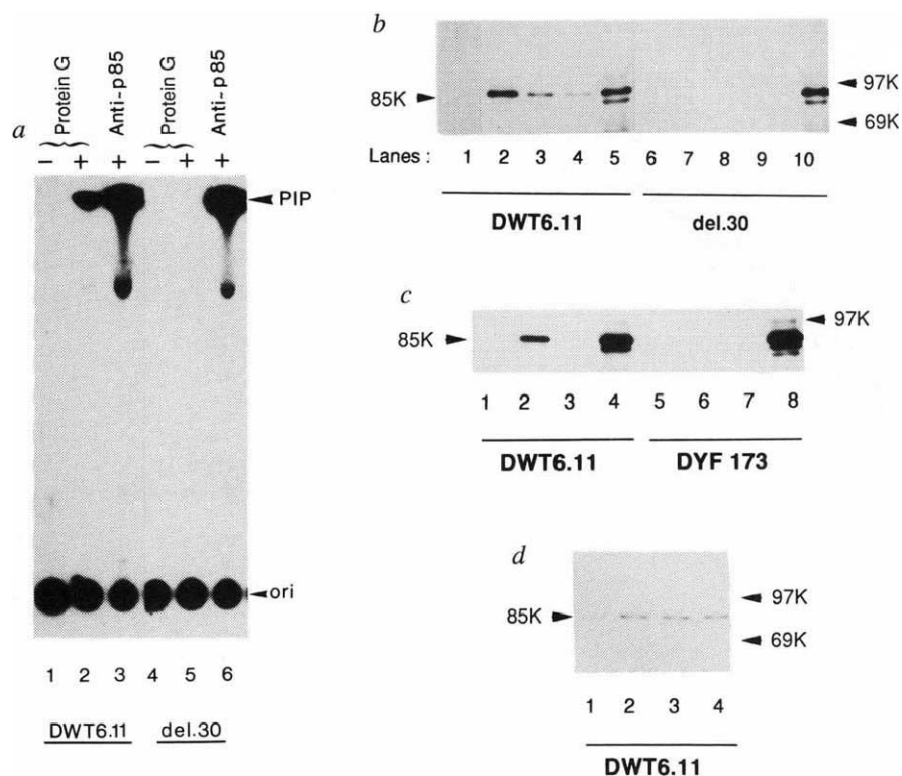
We transfected a murine CD3-TcR⁺ T-cell hybridoma with human CD28 full-length complementary DNA and with either a truncated form having the 30 C-terminal amino acids deleted just N-terminal of the ¹⁷³Tyr-X-X-Met motif (del.30) or a mutant form in which the tyrosine in the ¹⁷³Tyr-X-X-Met motif had been substituted by phenylalanine (DYF173). CD28⁺ wild-type (DWT6.11) and mutant stable transfected clones were activated by a monoclonal antibody against CD28. Phosphatidylinositol (PtdIns)-3-OH kinase was assayed on the CD28 immune complexes after cell lysis. After CD28 stimulation, 25% of the total precipitable PtdIns-3-OH kinase activity associated with the wild type (Fig. 1a, lane 2) but not with the del.30 mutant (Fig. 1a, lane 5). We next investigated the ability of the p85 subunit to mediate the binding of PtdIns-3OH kinase to CD28. The DWT6.11 and del.30 transfectants (Fig. 1b) or DYF173 (Fig. 1c) were stimulated by murine L cells transfected with human B7.1/CD80 (CD80⁺ L cells). Immunoblotting with anti-p85 antibodies showed that p85 was not associated with CD28 in unstimulated cells (Fig. 1b, lane 1). Stimulation of DWT6.11 but not del.30 (Fig. 1b, lanes 2 and 7) or DYF173 (Fig. 1c, lanes 2 and 6) cells by CD80⁺ L cells induces the association of p85 with CD28. This association was inhibited by preincubation with anti-B7.1/CD80 antibody (Fig. 1b, lane 3) or CTLA-4-Ig (Fig. 1b, lane 4 and c, lane 3). No p85 was detected in CD3 immune complexes after CD3 stimulation (data not shown). The effect of CD3 crosslinking on CD28 association with p85 was also tested. Activation of DWT6.11 by the murine anti-CD3 antibody 145.2C11, crosslinked or not with goat anti-mouse antiserum (Fig. 1d, lanes 4 and 3), did not modify the association of p85 with CD28 induced by CD28 stimulation (Fig. 1d, lane 2).

We investigated the ability of the SH2 domains of p85 to direct the interaction of p85 with CD28. CD28-transfected cells were stimulated with anti-CD28 antibody, lysed, and the lysates incubated with a bacterially expressed glutathione S-transferase (GST) fusion protein containing both the N- and C-terminal SH2 domains of p85 (GST-p85(N+C)SH2) immobilized on glutathione-Sepharose beads. Immunoblotting with the anti-CD28 antibody CD28.6 demonstrated that the p85 SH2 fusion protein coprecipitates with a band at M_r 44,000 (44K), which corresponds to CD28, following stimulation of DWT6.11 (Fig. 2a, lane 4). CD28 does not form similar complexes with SH2

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FIG. 1 a, PtdIns-3-OH kinase activity coprecipitates with CD28 DWT6.11 (lanes 1–3) and del.30 (lanes 4–6). Lanes: 1 and 4, unstimulated (–); 2 and 5, CD28 stimulated (+); 3 and 6, anti-p85 immunoprecipitation (IP). PIP, Phosphorylated phosphatidylinositol; ori, origin. b, p85 binding after stimulation by CD80⁺ L cells. DWT6.11 (lanes 1–5) and del.30 (6–10) stimulated with CD80⁺ L cells alone (lanes 2–4 and 7–9); or preincubated with anti-CD80 mAb (lanes 3 and 8) or CTLA-4-Ig (lanes 4 and 9); lanes 5 and 10, p85 immunoprecipitation. c, p85 subunit associates with the wild-type but not mutant huCD28 molecule. DWT6.11 and DYF173 mutant stimulated by CD80⁺ L cells without (lanes 2 and 6) or with CTLA-4-Ig (3 and 7); lanes 4 and 8, p85 immunoprecipitation. d, Effect of CD3 crosslinking on the association of p85 with CD28. DWT6.11 cells stimulated by CD28 mAb without (lane 2) or together with CD3 mAb (lane 3) or CD3 mAb and goat anti-mouse antiserum (lane 4).

METHODS. Wild-type (gift from B. Seed) and mutated human CD28 cDNA were inserted into the *Sall/BamHI* sites of the pH β act-pr-Neo vector and transfected^{13,14}. The del.30 deletion mutant and mutation of tyrosine 173 to phenylalanine have been described (F. P., manuscript in preparation). a, After activation, cells were lysed, immunoprecipitated and assayed for kinase as described¹⁵. b and c, DWT6.11, del.30 (b) or DYF173 (c) were stimulated with CD80⁺ L cells (A.T., manuscript in preparation), preincubated or not with anti-B7.1/CD80 mAb 104 (gift from J. Banachereau) or CTLA-4-Ig (gift from P. S. Linsley). Immunoprecipitation was done with CD28.2 mAb and protein G or p85 antiserum (UBI) and protein A (Pharmacia). Anti-p85 antiserum D was used



for immunoblotting, DWT6.11 were stimulated by CD28.2 mAb alone or in combination with CD3 mAb, without or with goat anti-mouse antiserum.

domains derived from phospholipase (PL) C- γ 1 (Fig. 2a, lane 6). The del.30 mutant failed to coprecipitate with the GST-p85(N+C)SH2 fusion protein (Fig. 2b, lane 4).

Because SH2 domains bind phosphotyrosyl-containing proteins, the coprecipitation of CD28 by the p85 SH2 domains indicates that CD28 might become tyrosine-phosphorylated following receptor activation. We therefore investigated the tyrosine phosphorylation of CD28 in response to the binding of its ligand B7.1/CD80. Stimulation of huCD28⁺ cells by CD80⁺ L cells demonstrated that CD28-B7.1/CD80 interaction induced the tyrosine phosphorylation of CD28 in DWT6.11 (Fig. 3a, lane 3 and b, lane 5) but not in del.30 (Fig. 3b, lane 12). Finally,

the activation of DWT6.11 hybridoma by either anti-CD28.2 antibody or CD80⁺ L cells resulted in the production of interleukin-2 (IL-2) (Fig. 4b). In contrast, neither del.30 (data not shown) nor DYF173 cells produce IL-2 after stimulation with anti-CD28 antibody or CD80⁺ L cells. DWT6.11, del.30 and DYF173 expressed comparably high levels of CD28 and were able to secrete IL-2 following activation by anti-CD3 antibody (Fig. 4a and b).

The production of phosphoinositides phosphorylated at the D-3 position of the inositol ring has been demonstrated in T cells following activation by IL-2, CD3, CD2, CD4 and CD28 (refs 9–12). We have shown that CD28 becomes tyrosine-phos-

FIG. 2 GST-SH2 (N- and C-terminal domains) of p85 coprecipitates CD28. DWT6.11 or del.30 cells were stimulated (+) or not (–) with CD28 mAbs, then the lysates were incubated with either protein G or the GST-p85(N+C)SH2 or GST-PLC γ 1(N+C)SH2 fusion proteins. Immunoblots were done with anti-CD28 mAb. DWT6.11 (a) or del.30 cells (b) were stimulated by CD28.2 mAb (a, lanes 2, 4 and 6; lanes 2 and 4) or left unstimulated (a, lanes 1, 3 and 5; b, lanes 1 and 3). Lysates were incubated with either protein G (a, lanes 1 and 2; b, lanes 1 and 2) or GST-p85(N+C) (a, lanes 3 and 4; b, lanes 3 and 4) or GST-PLC γ 1(N+C) (a, lanes 5 and 6).

METHODS. GST fusion proteins were prepared as described¹⁶ and immobilized on glutathione-Sepharose beads (Pharmacia). 10⁷ DWT6.11 or del.30 cells were stimulated by CD28.2 mAb (CD28.2 10 μ g ml⁻¹) or left unstimulated for 5 min at 37 °C, washed, lysed in NP-40 lysis buffer as described. Lysates (10⁷ cell equivalents per coprecipitation) were incubated with either 50 μ l protein G or 50 μ l GST-p85(N+C) or 50 μ l GST-PLC γ 1(N+C) for 1 h at 4 °C then washed in HNTG buffer (HEPES 20 mM pH 7, glycerol 10%, Triton X-100 0.1%, NaCl 150 mM, sodium vanadate 1 mM). Protein complexes were resolved by 10% SDS-PAGE, transferred to PVDF membranes and immunoblotted with the CD28 mAb CD28.6 (ref. 17). Proteins were detected by immunochemiluminescence (ECL, Amersham) and autoradiography.

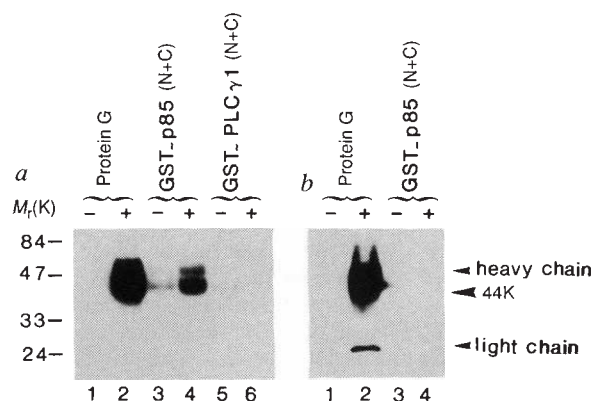
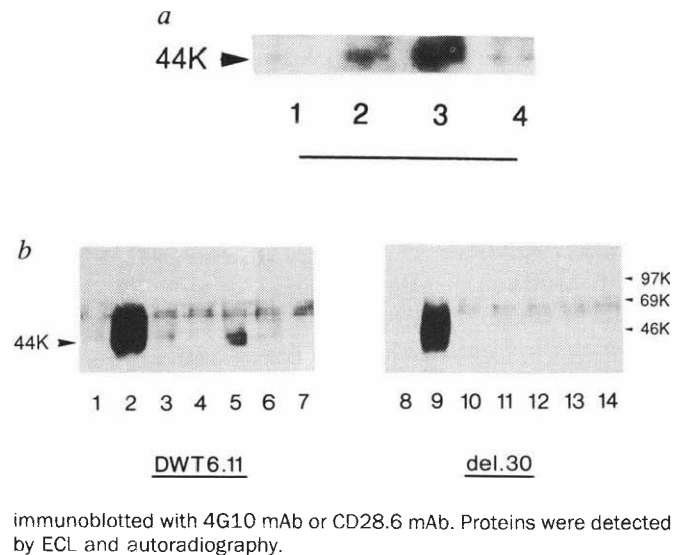
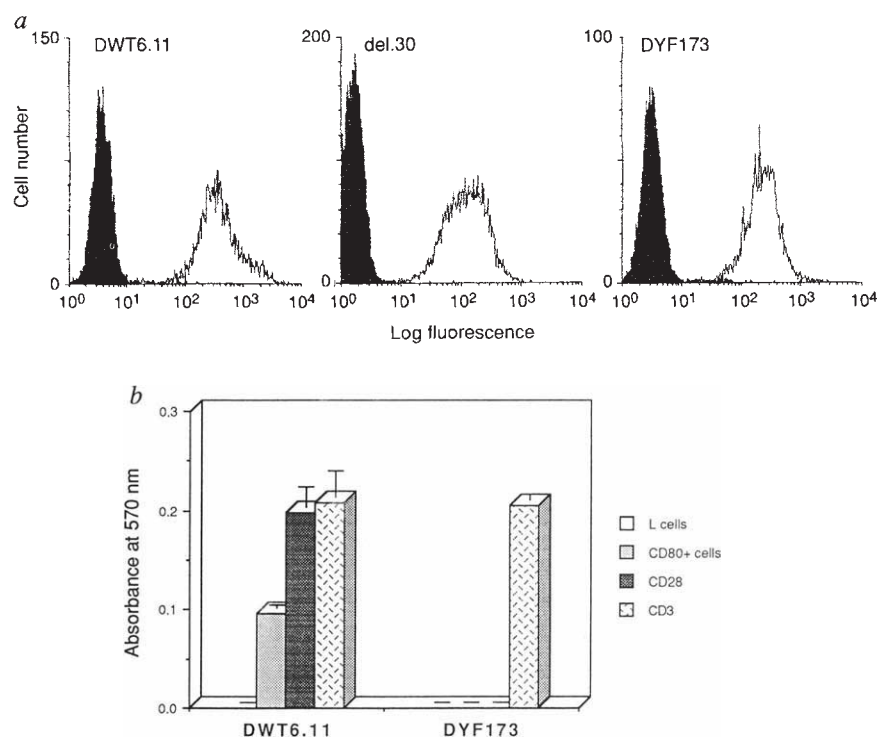


FIG. 3 Tyrosine phosphorylation of human CD28. **a**, DWT6.11 cells were stimulated with untransfected L cells or CD80⁺ L cells, lysed and CD28 molecules were immunoprecipitated with CD28 mAbs. Immunoblotting was done with anti-phosphotyrosine mAb. Lanes: 1, unstimulated cells; 2, untransfected L cells; 3, CD80⁺ L cells; 4, CD80⁺ L cells together with CTLA-4lg. **b**, DWT6.11 cells were stimulated with untransfected or CD80⁺ L cells. After lysis, tyrosine-phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine mAb and immunoblotted with CD28 mAb. DWT6.11 (lanes 1–7) or del.30 (lanes 8–14) were either not stimulated (lanes 1–3 and 8–10) or stimulated with L cells (lanes 4 and 11) or CD80⁺ L cells (lanes 5–7 and 12–14). CD28–B7.1/CD80 interaction was inhibited by the anti-B7.1/CD80 mAb 104 (lanes 6 and 13) or CTLA-4-Ig (lanes 7 and 14). Negative controls correspond to cell extracts from unstimulated CD28⁺ cells immunoprecipitated with protein G (lanes 1 and 8) or anti-phosphotyrosine mAb (lanes 3 and 10). Positive controls correspond to DWT6.11 cells unstimulated, lysed, immunoprecipitated with CD28.2 mAb and immunoblotted with CD28.6 mAbs (lanes 2 and 9). **M**, calibration is indicated on the right. **METHODS**. 10⁷ DWT6.11 cells or del.30 were incubated with 5 × 10⁶ L cells, 5 × 10⁶ CD80⁺ L cells with or without CTLA-4-Ig or the anti-B7.1/CD80 mAb 104, or left unstimulated. Cells were lysed, immunoprecipitated with CD28.2 mAb or 4G10 (anti-phosphotyrosine mAb; UBI) and protein-G beads. After SDS-PAGE and transfer, membranes were



immunoblotted with 4G10 mAb or CD28.6 mAb. Proteins were detected by ECL and autoradiography.

FIG. 4 Cell-surface expression and function of wild-type and mutant CD28. **a**, DWT6.11, del.30 and DYF173 cells were stained with CD28 mAb and analysed for CD28 cell-surface expression. **b**, DWT6.11 and DYF173 cells were stimulated with CD3 or CD28 mAbs or untransfected or CD80⁺ L cells. IL-2 production was measured by the proliferation of the IL-2-dependent cell line CTLL-2. **METHODS**. Expression of cell-surface CD28 was analysed by indirect immunofluorescence. DWT6.11, del.30 and DYF173 cells were incubated with saturating concentrations of CD28.2 mAb at 4 °C for 45 min, washed, then incubated with FITC-conjugated goat-anti-mouse Ig at 4 °C for 30 min (Jackson Laboratories). The samples were analysed by flow cytometry using a FACScan (Becton Dickinson). For IL-2 production, 10⁵ responding transfected cells were stimulated with either purified 145-2C11 (crosslinked anti-murine CD3; 10 µg ml⁻¹), CD28.2 (anti-human CD28.2; 30 µg ml⁻¹) mAbs or 5 × 10⁴ CD80⁺ or untransfected L cells in microtitre plates. After 24 h incubation, culture supernatants were collected and titrated by serial 2-fold dilutions for their ability to support proliferation of the IL-2-dependent murine T-cell line CTLL-2. Proliferation of CTLL-2 was measured by MTT (dimethylthiazol diphenyl tetrazolium bromide) assay (Sigma). The absorbance at 570 nm represents a supernatant dilution of 1 in 8.



phorylated and that up to 25% of the total cellular PtdIns-3-OH kinase activity inducibly associates with CD28 in response to B7.1/CD80 or antibody stimulation through the SH2 domains of the p85 subunit and a binding site within the carboxy tail of CD28 which includes the Tyr-X-X-Met motif at position 173. Analysis of del.30 and DYF173 stable transfectants shows that deletion or point mutation of the CD28 receptor abrogates PtdIns-3-OH kinase binding and IL-2 secretion, indicating that coupling the kinase to CD28 may relay a crucial feature of CD28 costimulatory function, perhaps by amplifying the T-cell receptor signal to augment the pool of kinase mobilized by the components of receptor complex. □

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Heterodimerization of the IL-2 receptor β - and γ -chain cytoplasmic domains is required for signalling

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THE interaction of interleukin-2 (IL-2) and IL-2 receptors critically regulates the T-cell immune response following antigen activation^{1,2}. IL-2 can signal through high or intermediate affinity receptors which contain IL-2R α (refs 3, 4) + β (refs 5–8) + γ (ref. 9) or β + γ chains, respectively. IL-2R γ is a common γ chain, γ_c , also shared by the IL-7 (ref. 10) and IL-4 (refs 11, 12) receptors, which when mutated results in X-linked severe combined immunodeficiency¹³. Using chimaeric receptor constructs together with monoclonal or bispecific antibodies we demonstrate here that IL-2 signalling requires ligand-induced extracellular-domain-mediated heterodimerization of the β - and γ_c -chain cytoplasmic domains. Anti-IL-2R α monoclonal antibodies trigger proliferation of cells transfected with chimaeric constructs in which the extracellular domains of IL-2R β and γ_c are replaced by that of IL-2R α . Other experiments using chimaeric constructs indicated that IL-2 binds monomerically and monovalently to IL-2R α and that the β -transmembrane domain is not required for receptor chain interactions. Finally, we provide a method for mapping residues in the γ_c cytoplasmic domain even in cells that constitutively express γ_c .

32D myeloid progenitor cells, which constitutively express murine γ_c (our unpublished observations), transduce an IL-2 specific signal¹⁴ when transfected with human IL-2R β ; the ability of murine γ_c to coordinate with human IL-2R β is not surprising given the high sequence homology between human and murine γ_c ^{15,16}. Although an IL-2R β mutant lacking 259 out of 286 amino acids of its cytoplasmic domain (β -ST¹⁷, Fig. 1a) can bind and mediate IL-2 internalization (ref. 17 and data not shown), it did not transduce an IL-2 signal (ref. 17 and Fig. 2a), demonstrating the key role of the IL-2R β cytoplasmic domain. Cells transfected with a chimaeric construct containing the ligand-binding domain of IL-2R α and the cytoplasmic domain of IL-2R β (denoted $\alpha/\alpha/\beta$ for α extracellular/ α transmembrane/ β cytoplasmic domains, Fig. 1a), expression of which was confirmed by flow cytometry (Fig. 1b) and western blotting (Fig. 1c), however, did not proliferate in response to IL-2, although they responded to IL-3 (Fig. 2a). Similarly, $\alpha/\beta/\beta$ (Fig. 1a) also did not transduce a proliferative signal (Fig. 2a), but β /CD4/ β (Fig. 1a) behaved like wild-type β (Fig. 2a). Thus, in contrast to the critical role of the transmembrane domains of T-cell antigen receptor components¹⁸, the β -transmembrane domain is not essential for mediating interactions with other receptor components.

The inability of $\alpha/\alpha/\beta$ and $\alpha/\beta/\beta$ to signal may have resulted from an inability to interact with endogenous γ_c . Three lines of evidence suggest that β - γ_c dimerization is mediated by the extracellular domain of each protein: truncation of IL-2R β ¹⁷ or γ_c ¹⁹ cytoplasmic domains does not interfere with IL-2 binding affinity or internalization; both full-length γ_c ⁹ and truncation mutants of γ_c retaining only six residues of the cytoplasmic domain can be coimmunoprecipitated in the presence of IL-2 by appropriate anti-IL-2R β antibodies (data not shown); and the IL-2R β transmembrane domain itself could be substituted without loss of activity.

We considered that the function of the IL-2R β and γ_c extracellular domains might be to mediate dimerization of their cytoplasmic domains. By extension, alternative means of inducing dimerization should also result in signal transduction. To test this hypothesis, 32D cells were transfected with β -ST + $\alpha/\alpha/\beta$. Although $\alpha/\alpha/\beta$ expression was low (Fig. 1b), cells proliferated in response to IL-2 as potently as β - or β /CD4/ β -transfected cells (Fig. 2b), indicating that the β -ST construct could provide the IL-2R β extracellular domain for dimerization with endogenous γ_c , and that binding of IL-2 would then allow the interaction of the β -ST + γ_c dimer with $\alpha/\alpha/\beta$, juxtaposing the cytoplasmic domains of IL-2R β and γ_c . Moreover, cells cotransfected with both $\alpha/\alpha/\beta$ and $\alpha/\gamma/\gamma$ were stimulated to proliferate by either 7G7/B6 or anti-Tac, two antibodies that bind to the IL-2R α extracellular domain and presumably induce both homodimerization and heterodimerization of the two chimaeric constructs (Fig. 3a). Cells transfected with $\alpha/\alpha/\beta$ or $\alpha/\gamma/\gamma$ alone were not activated by 7G7 or anti-Tac, suggesting that heterodimerization of these species was required. Although heterodimerization could not be demonstrated in the transfected 32D cells, possibly because of their low expression of $\alpha/\alpha/\beta$ (Fig. 1), heterodimers of the chimaeric constructs were identified by coprecipitation in COS-7 cells (Fig. 3b).

Proliferation of cells expressing $\alpha/\alpha/\beta$ + $\alpha/\gamma/\gamma$ was not induced by control RPC5 antibody or by IL-2 (Fig. 3a). High doses of IL-2 diminished anti-Tac-induced proliferation (Fig. 3c), consistent with the ability of IL-2 to inhibit anti-Tac binding^{20,21}. The fact that IL-2 only partially inhibited anti-Tac-induced proliferation is probably explained by the higher affinity of IL-2R α for anti-Tac than for IL-2 (dissociation constant, K_d , of 10^{-9} – 10^{-10} M¹ versus 10^{-8} M¹, respectively) and by the observation that even a low per cent occupancy of IL-2Rs is sufficient to induce significant proliferation, so that in a 20 h proliferation assay, enough anti-Tac could bind to the cells even with an excess of IL-2.

In many growth factor receptor systems, including those with receptor tyrosine kinases, ligand-induced dimerization is required for signalling²². Our data and evidence of homo- or heterodimerization of gp130 in IL-6 or leukaemia inhibitory factor signalling²³ support a model of ligand-induced dimerization as an initial step in signalling for the cytokine receptor superfamily. Some anti-receptor antibodies can induce dimerization and mimic the biological activity of certain growth factors^{24,25}. In the case of the IL-2 receptor, none of the antibodies reported is agonistic, presumably because none of them coordinates IL-2R β and γ_c . In contrast, a bispecific antibody recognizing both IL-2R α and IL-2R β (denoted bi-mAb)²⁶ induced proliferation of cells transfected with IL-2R β + $\alpha/\gamma/\gamma$ (Fig. 3d), whereas the bi-mAb inhibits IL-2-induced proliferation of normal T cells²⁶ by preventing IL-2 binding and thus IL-2-mediated dimerization of the β and γ_c cytoplasmic domains. Cells transfected with β + $\alpha/\gamma/\gamma$ were not activated by the simultaneous addition of Mik β 1 (anti- β) and 7G7/B6 (anti- α), even when a second tier goat anti-mouse antibody was added (Fig. 3d) suggesting that functional IL-2Rs appear to contain a heterodimer of β and γ_c rather than a higher-order aggregate of receptor chains.

We have shown that dimerization of β and γ_c cytoplasmic domains is necessary and sufficient for signalling (Fig. 4). We suggest that a properly engineered single-chain molecule con-

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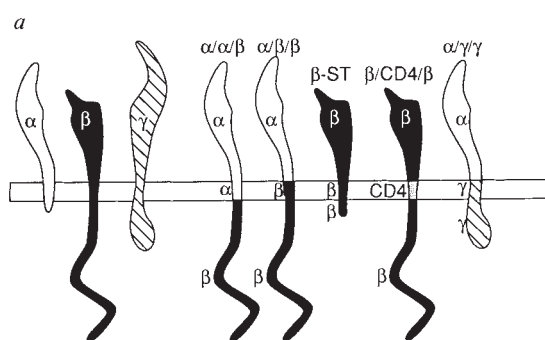
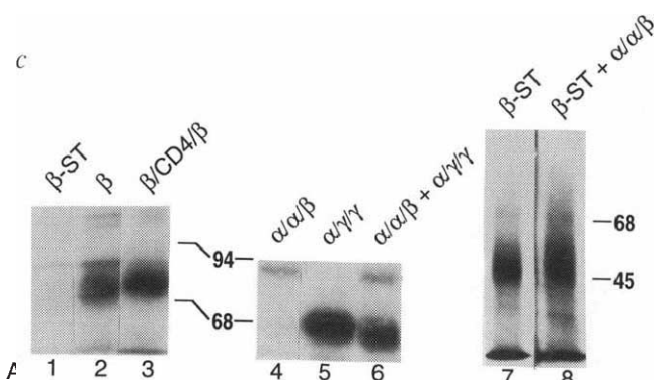
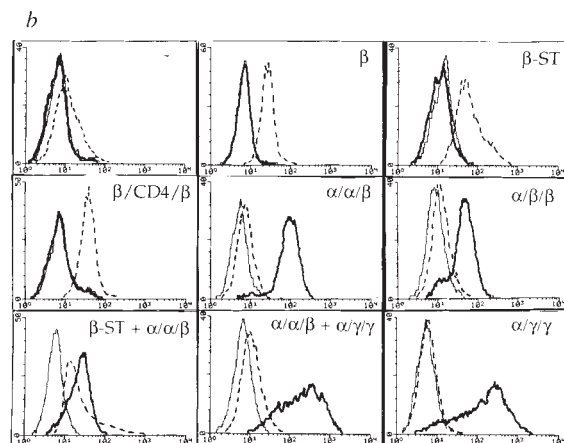


FIG. 1 a, Diagram of normal α , β , and γ chains and $\alpha/a/\beta$, $a/b/\beta$, β -ST, $\beta/CD4/\beta$, and $a/\gamma/\gamma$ chimaeric constructs. b, Flow cytometric analysis of representative clones of untransfected 32D cells (—) and cells transfected with β , β -ST, $\beta/CD4/\beta$, $a/a/\beta$, $a/b/\beta$, β -ST + $a/a/\beta$, $a/a/\beta$ + $a/\gamma/\gamma$, and $a/\gamma/\gamma$, stained with RPC5 (control IgG2a, thin line; Litton Bionetics), anti-Tac (anti-IL-2R α , thick line), and Mik- β 1 (anti-IL-2R β , broken line) mAbs shows appropriate cell surface expression of α and β epitopes. c, Western blotting of representative β -ST, β and $\beta/CD4/\beta$ transfectants using ErdA (anti-IL-2R β ; lanes 1–3) and of $a/a/\beta$, $a/b/\beta$, and $a/a/\beta$ + $a/\gamma/\gamma$ transfectants using R3134 (anti-IL-2R α ; lanes 4–6) polyclonal antisera and 125 I-protein A. The ErdA epitope(s) are in the cytoplasmic domain of IL-2R β distal to the β -ST truncation point; therefore, β -ST-transfected cells were surface labelled with Na 125 I, lysed and immunoprecipitated (IP) with #561 anti-IL-2R β mAb (lanes 7, 8). Less β -ST was expressed in β -ST + $a/a/\beta$ cells than in β -ST cells (Fig. 1b), but we have equalized signal intensities in lanes 7 and 8 to show they express the same β -ST mutant protein. Expected M_r s for constructs in c are: β and $\beta/CD4/\beta$ (75K); β -ST (45–50K); $a/a/\beta$ (85–90K), $a/\gamma/\gamma$ (65K).

METHODS. All constructs were subcloned in the expression vector pME18S 27 . Sequences were confirmed whenever synthetic oligonucleotides or PCR reactions were involved. pME18S- α was constructed by ligating the NciI to XbaI fragment (filled in at the NciI site) from the pIL-2R3 IL-2R α cDNA 3 to pME18S digested with EcoRI + XbaI, filled in at the EcoRI site. For pME18S- β , PCR was used to replace the 5' untranslated region of pBS β 28 with an EcoRI restriction site, followed by the Kozak translation start consensus sequence; the full coding region and 3' untranslated region was then cloned into pME18S between the EcoRI and XhoI sites (filled in only at the XhoI site). For pME18S- $a/a/\beta$, complementary oligonucleotides encoding the IL-2R α extracellular domain 3' of the BclI site, the IL-2R α transmembrane region, and the IL-2R β cytoplasmic domain 5' of the NcoI site were annealed, digested with BclI and NcoI, and joined by three-way ligation to the HindIII/BclI fragment from pME18S- α containing the SR α promoter and most of the IL-2R α extracellular domain and the NcoI/HindIII fragment from pME18S- β spanning most of the β cytoplasmic domain. For pME18S- $a/b/\beta$, the IL-2R α extracellular domain 3' of the IL-2R α AatII



site and the IL-2R β transmembrane and proximal IL-2R β cytoplasmic domains 5' of the IL-2R β AatII site were generated by two-step coupled PCR 29 and ligated into AatII-digested pME18S- $a/a/\beta$. For pME18S- $a/\gamma/\gamma$, two-step coupled PCR was used to generate the IL-2R α extracellular domain 3' of the NdeI site fused to the γ_c transmembrane and cytoplasmic domains (from pME18S- γ_c 30). This fragment was used to replace the NdeI to XbaI region of pME18S α . For pME18S- $\beta/CD4/\beta$, IL-2R β was mutated using the Biorad Mutagen kit and an oligonucleotide containing the CD4 transmembrane domain flanked by sequences that flank the β transmembrane domain. IL-2R constructs in pME18S were cotransfected with either pcDneo or TG76 (hygromycin B resistance plasmid) using electroporation (Bio-Rad Gene Pulser). Cells were placed under G418 and/or hygromycin B selection and clones selected by limiting dilution or cell sorting.

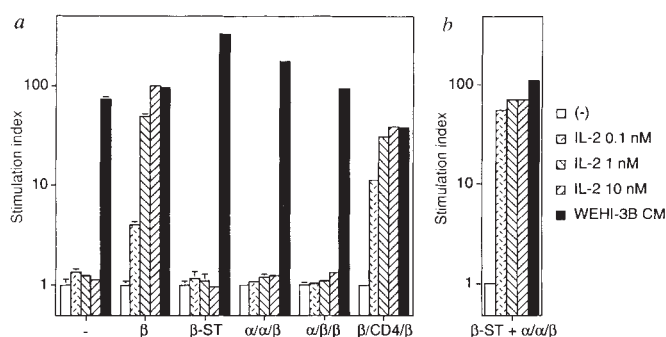


FIG. 2 Proliferation of 32D transfectants. a, 32D cells transfected with β -ST, $a/a/\beta$, or $a/b/\beta$ did not proliferate in response to IL-2 but cells transfected with wild-type β or $\beta/CD4/\beta$ were potentially stimulated by IL-2. IL-2 did not induce proliferation of β -ST or $a/a/\beta$ even at 100 nM (data not shown). b, Cells transfected with β -ST + $a/a/\beta$ proliferated in response to IL-2. Data are presented as stimulation indexes (fold increase in proliferation relative to basal proliferation in the absence of stimuli). For each construct, at least two independent clones were analysed with similar results in multiple independent experiments. Shown are means $\pm$ s.d. of triplicate data points from representative experiments; s.d.s are not shown where too small to be visible. METHODS. Cells were maintained in RPMI 1640 supplemented with 10% FBS and 5% WEHI-3B conditioned medium (CM) as an IL-3 source 14 . Cells (2×10^4 in 200 μ l) were cultured for 16 h in triplicate in 96-well microculture plates (Corning) with 0–10 nM recombinant human IL-2 (Cetus Corp.), and/or 5% WEHI-3B CM followed by a 4 h pulse with 0.5 μ Ci of [3 H]thymidine (New England Nuclear).

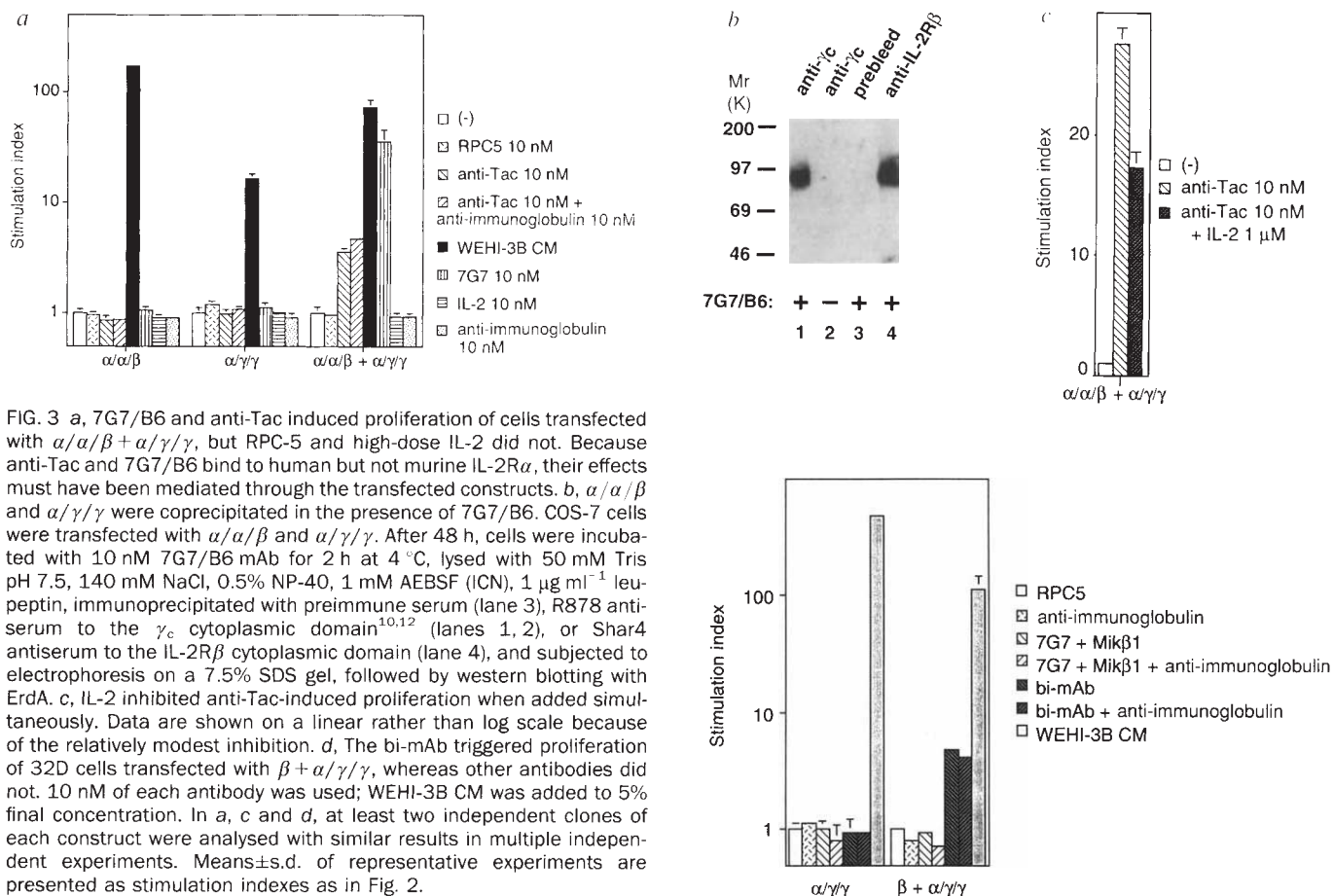
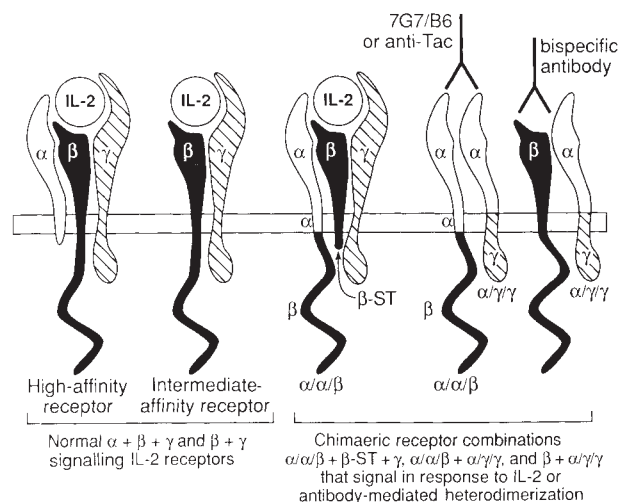


FIG. 3 **a**, 7G7/B6 and anti-Tac induced proliferation of cells transfected with $\alpha/\alpha/\beta + \alpha/\gamma/\gamma$, but RPC-5 and high-dose IL-2 did not. Because anti-Tac and 7G7/B6 bind to human but not murine IL-2R α , their effects must have been mediated through the transfected constructs. **b**, $\alpha/\alpha/\beta$ and $\alpha/\gamma/\gamma$ were coprecipitated in the presence of 7G7/B6. COS-7 cells were transfected with $\alpha/\alpha/\beta$ and $\alpha/\gamma/\gamma$. After 48 h, cells were incubated with 10 nM 7G7/B6 mAb for 2 h at 4 °C, lysed with 50 mM Tris pH 7.5, 140 mM NaCl, 0.5% NP-40, 1 mM AEBSF (ICN), 1 μ g ml⁻¹ leupeptin, immunoprecipitated with preimmune serum (lane 3), R878 antiserum to the γ_c cytoplasmic domain^{10,12} (lanes 1, 2), or Shar4 antiserum to the IL-2R β cytoplasmic domain (lane 4), and subjected to electrophoresis on a 7.5% SDS gel, followed by western blotting with ErdA. **c**, IL-2 inhibited anti-Tac-induced proliferation when added simultaneously. Data are shown on a linear rather than log scale because of the relatively modest inhibition. **d**, The bi-mAb triggered proliferation of 32D cells transfected with $\beta + \alpha/\gamma/\gamma$, whereas other antibodies did not. 10 nM of each antibody was used; WEHI-3B CM was added to 5% final concentration. In **a**, **c** and **d**, at least two independent clones of each construct were analysed with similar results in multiple independent experiments. Means $\pm$ s.d. of representative experiments are presented as stimulation indexes as in Fig. 2.

taining both β and γ_c cytoplasmic domains or spontaneously dimerizing β and γ_c constructs might mediate an IL-2-independent constitutive proliferative signal. These results indicate that the extracellular domains of IL-2R β and γ_c allow IL-2 mediated heterodimerization whereas the cytoplasmic domains contain information necessary and sufficient for signalling, and trigger signalling when heterodimers but not when homodimers are formed. Several additional conclusions can be made. First, the

activation of a combination of $\alpha/\alpha/\beta$ and $\alpha/\gamma/\gamma$ by anti- α antibodies (Fig. 3a) indicates that IL-2 and the extracellular domains of β and γ_c are dispensable to the ability of β and γ_c to mediate a proliferative signal. Second, the activity of β /CD4/ β (Fig. 2) suggests that important interactions are not mediated by the β -transmembrane domain. Third, the inability of IL-2 to activate $\alpha/\alpha/\beta + \alpha/\gamma/\gamma$ suggests that IL-2 binds monomerically and monovalently to the IL-2R α extracellular domain. Finally,

FIG. 4 Diagram of normal high-affinity ($\alpha + \beta + \gamma$) and intermediate-affinity ($\beta + \gamma$) IL-2Rs and artificial chimaeric receptor combinations that signalled in response to IL-2 (β -ST + $\alpha/\alpha/\beta + \gamma$), anti-IL-2R α antibodies ($\alpha/\alpha/\beta + \alpha/\gamma/\gamma$), or the bi-mAb ($\beta + \alpha/\gamma/\gamma$). Juxtaposing the β and γ cytoplasmic domains appears both necessary and sufficient for signalling.



the use of chimaeric constructs provides a system for mapping important residues for γ_c and IL-2R β cytoplasmic domains even in cells expressing γ_c and IL-2R β . This type of approach should be widely applicable to other cytokine receptors. □

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Cytoplasmic domains of the interleukin-2 receptor β and γ chains mediate the signal for T-cell proliferation

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THE interleukin-2 receptor (IL-2R) consists of three distinct chains (α , β , γ) which bind IL-2 and generate a proliferative signal in T cells¹. To define the mechanism of receptor activation, chimaeric receptors were constructed from the intracellular region of either IL-2R β or IL-2R γ and the extracellular region of c-kit, a receptor tyrosine kinase that homodimerizes on binding stem cell factor (SCF)². We report here that binding of SCF to the β -chain chimaera induced proliferation of the pro-B-cell line BA/F3, but not T cells. But in T cells expressing both the β - and γ -chain chimaeras, SCF induced proliferation and tyrosine phosphorylation characteristic of the native IL-2R signal. Chimaeric IL-2 receptor β and γ chains constructed with the heterodimeric extracellular regions of the granulocyte-macrophage colony stimulating factor receptor (GM-CSFR) also provided the IL-2R signal. Thus, heterodimerization of the cytoplasmic domains of IL-2R β and γ appears necessary and sufficient for signalling in T cells.

On the basis of homology to other haematopoietic receptors, such as the IL-6 receptor β chain³, we and others⁴ speculated that IL-2R signalling might reflect ligand-induced homodimerization of IL-2R β , resulting in activation of associated tyrosine kinases and substrate phosphorylation^{5,6}, with the α and γ chains contributing primarily to ligand binding and internalization. Therefore, we constructed a chimaeric receptor (kit/2 β) containing the intracellular region of IL-2R β and the extracellular region of c-kit (Fig. 1a). Expression of kit/2 β in the pro-B cell line BA/F3 was adequate for proliferation and long-term growth in response to SCF (Fig. 2a). However, upregulation of IL-2R α expression, an IL-2R-mediated response of

lymphocytes⁷, did not occur (Fig. 3a). Therefore, a second chimaeric receptor (kit/2 γ) was constructed using the intracellular region of IL-2R γ (Fig. 1a). SCF induced proliferation and expression of IL-2R α only in BA/F3 cells coexpressing kit/2 β and kit/2 γ (Figs 2a and 3b).

These results suggested that the authentic IL-2R signal might require direct interaction of the β and γ chains and that the proliferative signal observed in BA/F3 cells expressing kit/2 β alone might mimic events following homodimerization of homologous single-chain haematopoietic receptors such as the EPO-R⁸. Therefore, kit/2 β and kit/2 γ were introduced into the IL-2-dependent T-cell line CTLL2, which unlike BA/F3 cells does not respond to EPO-R-mediated signals (B.H.N., unpublished data). SCF did not induce proliferation of CTLL2 cells expressing kit/2 β , kit/2 γ or wild-type c-kit alone (Figs 2b and 3c). However, CTLL2 cells coexpressing kit/2 β and kit/2 γ proliferated and could be propagated in response to SCF.

The proliferative response of CTLL2 cells expressing chimaeric IL-2R β and γ chains suggested IL-2R α was not essential for signalling. However, antibodies to the α chain inhibit proliferation of primary T cells⁹, which differ from CTLL2 in requiring antigen stimulation for IL-2 responsiveness. Therefore, chimaeric receptors were introduced into the CD4⁺ T-helper clone D10¹⁰. Antigen-stimulated D10 cells expressing kit/2 β , kit/2 γ or c-kit alone did not respond to SCF; however, cells coexpressing kit/2 β and kit/2 γ proliferated and could be propagated with SCF in place of IL-2. The proliferative response to SCF, like that to IL-2, required prior antigen stimulation (Fig. 2c). These experiments exclude an obligatory cytoplasmic role for IL-2R α in signalling; instead IL-2R α may stabilize interactions between the β and γ chains by increasing receptor affinity for IL-2¹¹.

The major substrate of IL-2-induced tyrosine phosphorylation in T cells is a 97K protein, with additional minor substrates of about 55, 92, 100 and 130K^{12,13}. These proteins were phosphorylated in response to SCF in D10 cells coexpressing kit/2 β and kit/2 γ (Fig. 4a). Phosphorylation of kit/2 β and kit/2 γ was also observed, consistent with phosphorylation of IL-2R β and γ in response to IL-2^{14,15}. SCF did not induce detectable tyrosine phosphorylation in D10 cells expressing kit/2 β or kit/2 γ individually (Fig. 4a), but in BA/F3 cells, expressing kit/2 β alone induced tyrosine phosphorylation of kit/2 β and five other substrates (Fig. 4b). Thus, although kit/2 β can mediate tyrosine phosphorylation and proliferation in permissive cell lines like

BA/F3, the results in T cells suggest that phosphorylation and proliferation characteristic of the IL-2R signal requires a direct interaction between the β and γ chains.

To confirm that IL-2R signalling results from heterodimerization of the β and γ chains, alternative chimaeric IL-2R chains were constructed with extracellular regions from the α and β chains of the heterodimeric GM-CSF receptor¹⁶ (Fig. 1b). Neither the parental CTLL2 T cell line nor transfectants expressing the chimaeric receptors GM α /2 γ or GM β /2 β individually proliferated in response to GM-CSF (Fig. 2d). However, cells coexpressing GM α /2 γ and GM β /2 β proliferated and could be propagated in response to GM-CSF. Moreover, CTLL2 cells coexpressing kit/2 β , which binds SCF (Fig. 2a), and GM α /2 γ , which binds GM-CSF (data not shown) did not proliferate in response to GM-CSF and SCF (Fig. 2d), implying each ligand must simultaneously bind both a β - and γ -chain chimaera to induce proliferation.

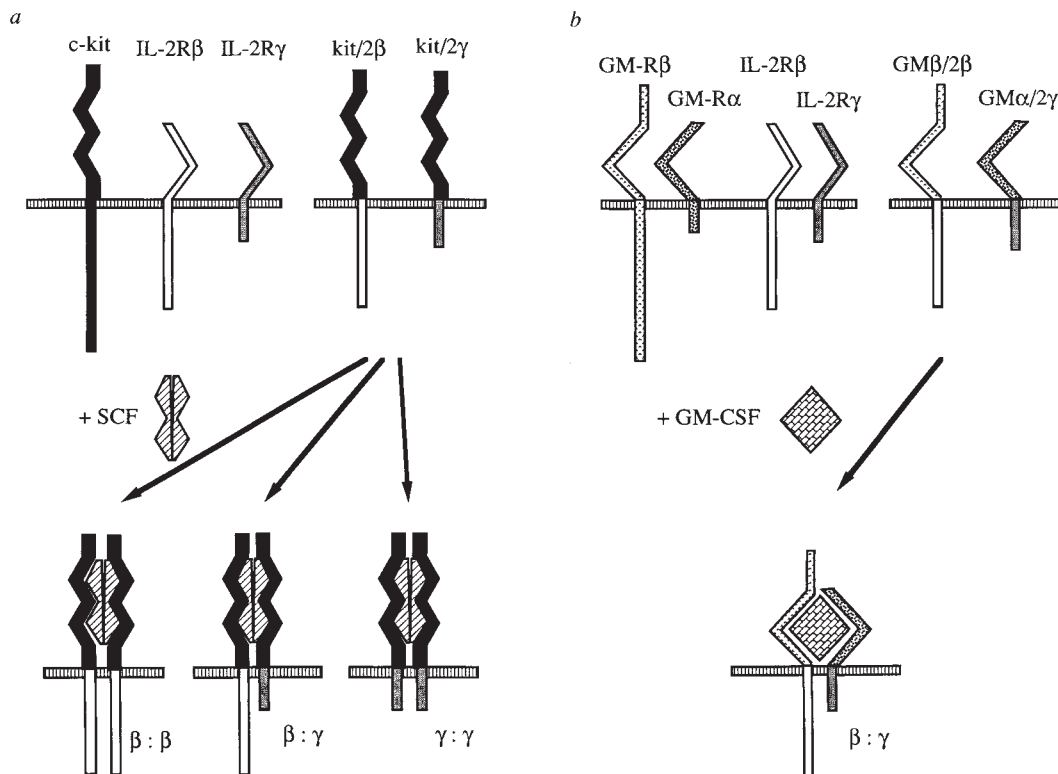
Chiba *et al.*¹⁷ have recently proposed that the extracellular regions of haematopoietic receptors determine the specificity of tyrosine-kinase activation, whereas our results implicate the

cytoplasmic regions of IL-2R β and γ . However, Chiba *et al.* did not take into account the signalling function of IL-2R γ demonstrated here, and in fibroblasts¹⁸. Thus, the two tyrosine phosphorylation patterns they described in BA/F3 cells probably reflected heterodimerization with endogenous IL-2R γ of their chimaeric receptors containing extracellular IL-2R β domains versus homodimerization of the intracellular region of their chimaeric receptors containing extracellular EPO-R domains (as observed with kit/2 β).

Our conclusion that heterodimerization of the intracellular regions of IL-2R β and γ is necessary and sufficient for signalling probably extends to the IL-4 and IL-7 receptors, which have unique β chains that associate with IL-2R γ ¹⁹⁻²², and to the heterodimeric IL-3, IL-5 and GM-CSF receptors, which share cytoplasmic homology with IL-2R β and γ ^{23,24}. Our results suggest that haematopoietic receptors function similarly to receptor tyrosine kinases²⁵. The role of extracellular regions is to bind cytokine and induce dimerization of the intracellular domains, resulting in activation of tyrosine kinases and assembly of the signal transduction complex. □

FIG. 1 Schematic representation of chimaeric IL-2 receptors undergoing ligand-induced dimerization. a, The extracellular region of murine c-kit and the intracellular regions of human IL-2R β or IL-2R γ were used to construct chimaeric receptors designated kit/2 β and kit/2 γ , respectively. Binding of the SCF homodimer to the extracellular portion of kit/2 β and/or kit/2 γ potentially induces kit/2 β homodimers (β : β), kit/2 γ homodimers (γ : γ) or kit/2 β and kit/2 γ heterodimers (β : γ). b, The extracellular region of the human GM-CSFR α or β chains and the intracellular regions of human IL-2R β or IL-2R γ were used to construct chimaeric receptors designated GM β /2 β and GM α /2 γ , respectively, which are predicted to heterodimerize on binding GM-CSF.

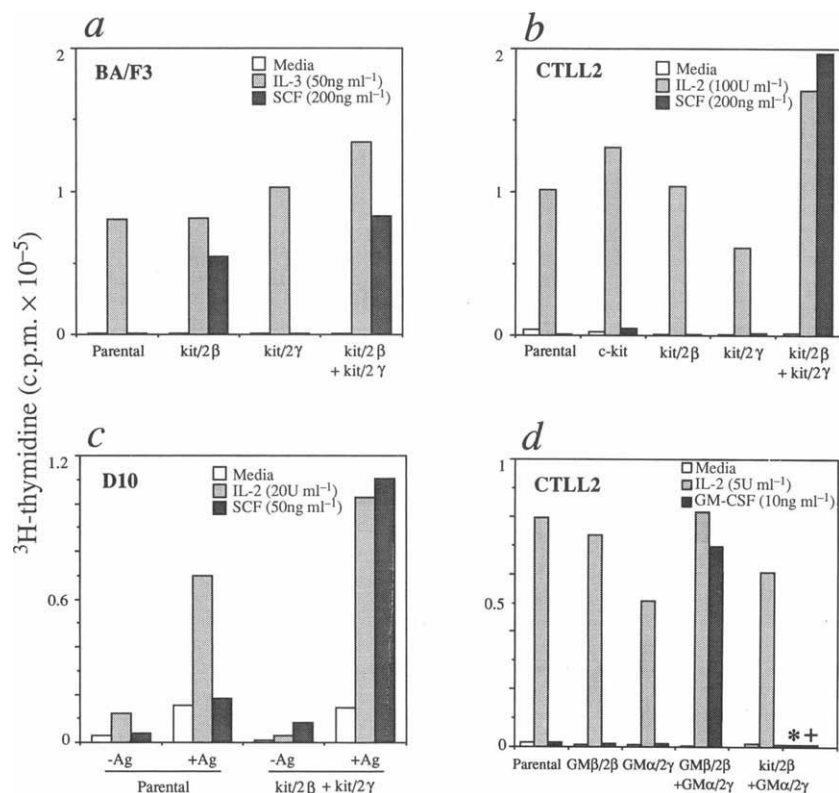
METHODS. A *KpnI*-blunted *Apal* fragment of cDNA encoding murine c-kit and *HincII*-*Bam*H1 fragment of cDNA encoding human IL-2R β were cloned in tandem into the plasmid pBluescript SK⁻ (Stratagene). An exact fusion between the extracellular region of c-kit and transmembrane region of IL-2R β was generated by site-directed loop-out mutagenesis (BioRad) using the fusion primer GCCGAGCCACGGAATGTGGGCCTGGATTG in which the underlined bases are complementary to IL-2R β and the remainder to c-kit. The transmembrane and cytoplasmic regions of human IL-2R γ were derived by reverse-transcription polymerase chain reaction (PCR) using mRNA from a human CD8⁺ T cell clone and primers with terminal *HincII* and *Bam*H1 restriction enzyme sites. The amplified product was cloned downstream of the c-kit cDNA in pBluescript SK⁻ in the same manner as IL-2R β . The c-kit extracellular region was fused to the IL-2R γ transmembrane region by overlap extension PCR²⁶ using the complementary fusion primers TGCAACAGGAAAGGGTGGGCCTGGATTG and CAA-



ATCCAGGCCACCCCTTCTGTTTGCA, in which the underlined bases are complementary to IL-2R γ and the remainder to c-kit. GM β /2 β and GM α /2 γ were constructed by a similar strategy using *Xho*I-blunted *Eco*R1 fragments of cDNA encoding GM-CSFR α or GM-CSFR β (KH97). Exact fusions between extracellular and transmembrane regions were made by overlap extension PCR using the complementary fusion primers TGCAACAGGAAAGGGCCGTCGTCAGAAC and GGTCTGACGACGGCCCTTCTGTTTGCA for GM α /2 γ , and GCCGAGCCACGGAATCGACTCGGTGTCCCA and TGGGACACCGAGTCGATTCGGTGGCTCGGC for GM β /2 β . Fusion sites and flanking regions were checked by standard DNA sequencing methods. kit/2 β , wild-type c-kit and GM β /2 β cDNAs were cloned into the eukaryotic expression vector pHAPr-1-neo²⁷, which confers resistance to neomycin, and kit/2 γ and GM α /2 γ cDNAs into a modified form of this vector conferring resistance to hygromycin.

FIG. 2 Activation of the IL-2R β cytoplasmic region is adequate for proliferation of BA/F3 cells, but the authentic IL-2R proliferative signal measured in T cells requires an interaction between the cytoplasmic domains of IL-2R β and γ . **a**, IL-3- and SCF-induced proliferative responses of the parental BA/F3 pro-B cell line and transfectants expressing c-kit/IL-2R chimaeric chains kit/2 β , kit/2 γ , or coexpressing kit/2 β and kit/2 γ . **b**, IL-2- and SCF-induced proliferative responses of the parental CTLL2 T cell line and transfectants expressing c-kit, kit/2 β , kit/2 γ , or coexpressing kit/2 β and kit/2 γ . **c**, IL-2- and SCF-induced proliferative responses of the parental D10 T-cell clone and transfectants coexpressing kit/2 β and kit/2 γ with (+Ag) or without (-Ag) antigen stimulation. **d**, IL-2 and GM-CSF-induced proliferative responses of the parental CTLL2 T cell line and transfectants expressing GM β /2 β or GM α /2 γ , or coexpressing GM β /2 β and GM α /2 γ or kit/2 β and GM α /2 γ . In the two rightmost columns, cells coexpressing kit/2 β and GM α /2 γ were incubated with 100 ng ml $^{-1}$ SCF with (plus) or without (asterisk) 500 ng ml $^{-1}$ GM-CSF. These concentrations of SCF and GM-CSF were adequate for saturable binding.

METHODS. CTLL2 cells were maintained in Click's media (Altick Enterprises) containing 100 units ml $^{-1}$ human IL-2 (Hoffman LaRoche), 10 mM HEPES, 0.14% sodium bicarbonate, 1% glutamine, 1% penicillin/streptomycin and 10% fetal calf serum (FCS). D10 and BA/F3 cells were maintained as described^{10,28}. Murine SCF and human GM-CSF were gifts from Immunex. Plasmids were introduced into lymphocytes by electroporation, and stably transfected lines were selected for resistance to G418 (1 mg ml $^{-1}$ for CTLL2 and BA/F3 transfectants and 0.5 mg ml $^{-1}$ for D10 transfectants; Gibco/BRL) and/or hygromycin B (0.25 mg ml $^{-1}$ for CTLL2, 0.5 mg ml $^{-1}$ for BA/F3 and 0.125 mg ml $^{-1}$ for D10 transfectants; Sigma). Receptor expression by all cells used in these analyses was confirmed by flow cytometry using the antibodies ACK-2 (Gibco/BRL) for c-kit-derived receptors and anti-GM-CSFR α -M1 (Immunex) for GM α /2 γ , or by reverse-transcriptase PCR for GM β /2 β . Moreover, for every cell line expressing a single chimaeric receptor, or mismatched chimaeric receptors (**d**), the possibility that the failure to proliferate in response to SCF or GM-CSF was due to inadequate receptor expression was excluded by introduction of the appropriate complementary chimaeric receptor and establishment of long-term growth on SCF or GM-CSF. Binding of GM-CSF to cells expressing GM α /2 γ with



or without GM β /2 β was assessed by flow cytometry with phycoerythrin-conjugated human GM-CSF (R&D Systems). For proliferative assays, CTLL2 and BA/F3 cells were washed twice with RPMI and incubated for 24 h in a 96-well plate at 4,000 (CTLL2) or 2,000 (BA/F3) cells per well in their respective media with the indicated concentrations of cytokines. For the final 4 h, each well contained 2.5 μCi ^3H -thymidine. Thymidine incorporation was quantified by liquid scintillation counting. D10 cells were rested for 7 days without antigen or added cytokines in the presence of a 10-fold excess of C3H splenocytes. On day 8, cells were washed once with RPMI and additional splenocytes were added with or without cognate antigen (conalbumin A, 200 μg ml $^{-1}$; Sigma) as described¹⁰ in the presence of the anti-IL-4 receptor antibody M1²⁹ (5 μg ml $^{-1}$; Immunex) to block IL-4-mediated autocrine growth. Twenty-four hours later, a proliferative assay was done as described above except 20,000 cells were plated per well, M1 antibody was included at 5 μg ml $^{-1}$, and ^3H -thymidine was present for 8 h.

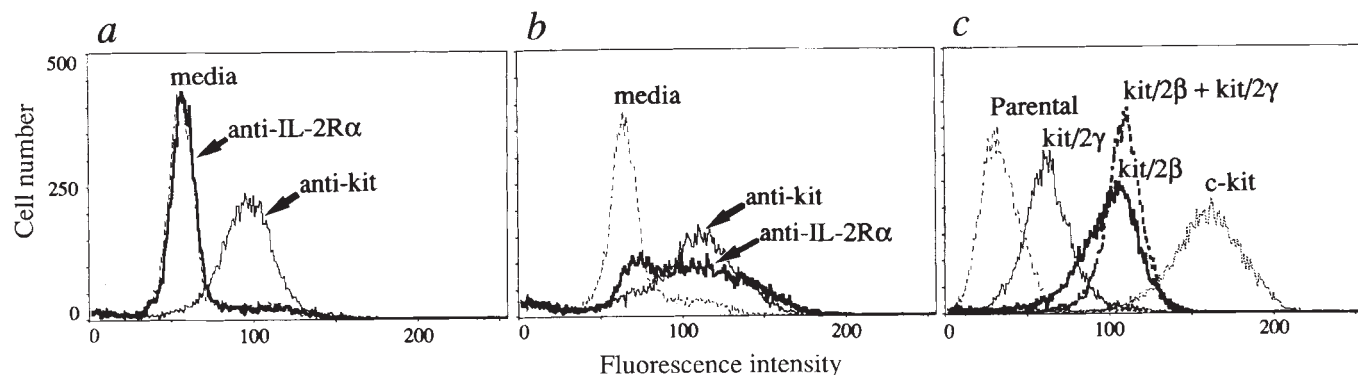


FIG. 3 Flow cytometric analysis of receptor expression on BA/F3 and CTLL2 transfectants. **a**, BA/F3 cells expressing kit/2 β and stimulated with SCF fail to express endogenous IL-2R α , whereas **b**, BA/F3 cells coexpressing kit/2 β and kit/2 γ express endogenous IL-2R α in response to SCF. Cells from **a** and **b** stimulated with IL-3 did not express IL-2R α (data not shown). **c**, kit/2 β , kit/2 γ and wild-type c-kit are expressed at high levels on CTLL2 transfectants but not the parental line.

METHODS. BA/F3 cells transfected with kit/2 β or with kit/2 β and kit/

2 γ were grown on IL-3 or SCF and immunostained with the rat antibody 7D4³⁰ specific for the extracellular region of murine IL-2R α or with the rat antibody ACK-2 (Gibco/BRL) specific for the extracellular region of murine c-kit or with media alone, followed by secondary staining with fluorescein-conjugated anti-rat IgG antibody (Tago). CTLL2 cells transfected with kit/2 β , kit/2 γ , wild-type c-kit, or both kit/2 β and kit/2 γ were immunostained with ACK-2 as above to assess receptor expression.

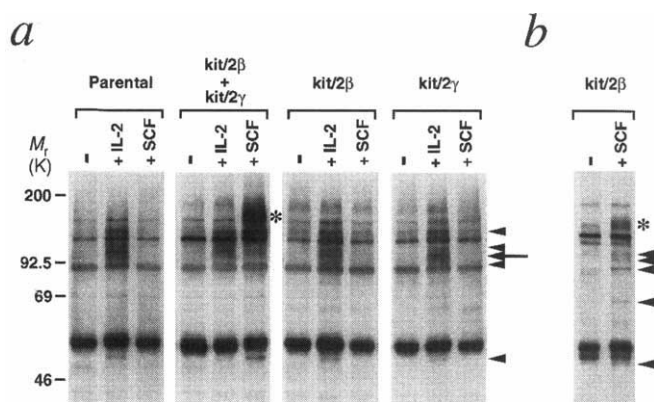


FIG. 4 Tyrosine phosphorylation in response to cytokine stimulation of D10 and BA/F3 cells expressing chimaeric receptors. *a*, Western blot analysis with anti-phosphotyrosine antibody of the parental D10 cell line (lanes 1–3) and transfected D10 lines coexpressing kit/2 β and kit/2 γ (lanes 4–6), or expressing only kit/2 β (lanes 7–9) or kit/2 γ (lanes 10–12). Cells were initially activated with antigen and then were left unstimulated (–) or were stimulated with IL-2 or SCF as indicated. The arrow indicates the position of a 97K protein that is the major substrate of tyrosine phosphorylation induced by IL-2 or, in lane 6, by SCF. Arrowheads indicate the positions of four minor substrates. The asterisk refers to 140–160K proteins phosphorylated in response to SCF which resolved into two species on low-density gels and corresponded to kit/2 β and kit/2 γ by molecular mass and immunoprecipitation with ACK-2 antibody. *b*, Western blot analysis with anti-phosphotyrosine antibody of BA/F3 transfectants expressing kit/2 β and left unstimulated (–) or stimulated with SCF as indicated. Arrowheads indicate the positions of five proteins phosphorylated in response to SCF, and the asterisk refers to a 150K phosphoprotein corresponding to kit/2 β .

METHODS. Parental or transfected cell lines were washed twice with RPMI 1640 and incubated at 10^6 cells per ml in culture media for 4 h without added cytokine. During the last 30 min, 50 μ M sodium orthovanadate was added to the medium, and cells were then stimulated for 20 min with human IL-2 (100 units ml $^{-1}$) or murine SCF (200 ng ml $^{-1}$). Cells were solubilized at 10^8 cells ml $^{-1}$ in 1% Triton-X100, 50 mM Tris, 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM PMSF, pH 7.5. Extracts from 4×10^5 D10 cells or 2×10^5 BA/F3 cells were boiled for 5 min in SDS sample buffer and applied to an 8% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose and immunoblotted in 0.1 M Tris, 0.9% NaCl, 1% BSA, 0.05% Tween 20 (Sigma), pH 7.5, with monoclonal anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology) followed by horseradish peroxidase-conjugated goat anti-mouse antibody (Gibco/BRL). Detection was by enhanced chemiluminescence (Amersham).

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Somatostatin-induced inhibition of neuronal Ca $^{2+}$ current modulated by cGMP-dependent protein kinase

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NEUROTRANSMITTER release is frequently regulated by peptides that modulate neuronal calcium channels. Whole-cell recordings show that the ion permeability¹ and voltage dependence² of these channels are controlled by a membrane-associated pathway involving GTP-binding proteins. Here we use perforated-patch recordings to show that, in addition to this pathway, the peptide somatostatin inhibits the calcium current in chick ciliary ganglion neurons by a second soluble pathway involving a cyclic GMP-dependent protein kinase (cGMP-PK). This somatostatin inhibition of Ca $^{2+}$ current did not desensitize and was not characterized by the slowing of Ca $^{2+}$ -current activation (kinetic slowing) observed in whole-cell recordings. When cGMP-PK was inhibited, somatostatin inhibition of Ca $^{2+}$ current resembled that observed with whole-cell recordings. cGMP agonists mimic the effect of somatostatin only in perforated patch recordings. An endogenous cGMP-PK therefore forms part of the mechanism by which somatostatin induces a sustained inhibition of neuronal calcium channels.

The effect of 100 nM somatostatin on voltage-gated Ca $^{2+}$ current recorded from ciliary ganglion neurons with whole-cell recording techniques is illustrated in Fig. 1*a*. Somatostatin rapidly reduced voltage-gated Ca $^{2+}$ current by $46.5 \pm 2.9\%$ (mean $\pm$ s.e.m.; $n = 10$). This inhibition was associated with a slowing of Ca $^{2+}$ -current activation kinetics, measured as the per cent inhibition at the time when control currents peak versus the inhibition observed at the end of the voltage pulse ($46.5 \pm 2.9\%$ versus $30.3 \pm 3.3\%$, $P < 0.002$). This phenomenon, hereafter referred to as kinetic slowing, is characteristic of peptide-mediated inhibition described elsewhere and has been hypothesized to represent voltage-dependent inhibition mediated through a membrane-delimited pathway triggered by a GTP-binding protein (G protein)^{2,9}. In contrast, using perforated-patch recordings, the somatostatin-mediated inhibition was not associated with kinetic slowing ($51.9 \pm 2.6\%$, $n = 17$, inhibition at the peak versus $43.5 \pm 2.3\%$ at the end of the pulse; Fig. 1*b*). Kinetic slowing, however, reappeared following somatostatin application in the presence of a specific inhibitor of cGMP-PK, Rp-8(4-chlorophenylthio)-guanosine-3',5'-cyclic monophosphorothioate (Rp-8-pCPT-cGMPS; ref. 10, and E. Butt and U. Walter, manuscript in preparation). After a 15-min exposure to 100 μ M Rp-8-pCPT-cGMPS, somatostatin-induced inhibition of Ca $^{2+}$ current

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(Fig. 1c) displayed kinetic slowing ($60.3 \pm 3.2\%$ inhibition at the peak; $43.4 \pm 4.6\%$ at the end of the pulse; $n=9$, $P<0.01$) like that observed with whole-cell recordings (Fig. 1a).

Previously, whole-cell measurements of neuropeptide-modulated Ca^{2+} current have demonstrated that this inhibition was voltage-dependent^{1,2,5-9}. Specifically, the magnitude of inhibition decreased as the voltage step used to activate Ca^{2+} current became more depolarized. These observations have led to a model for Ca^{2+} -current inhibition in which the membrane-delimited pathway produces a shift in the voltage dependence of Ca^{2+} -channel activation². This 'willing-reluctant' model has also been proposed to explain the kinetic slowing often observed following neuropeptide-mediated Ca^{2+} -current inhibition². As we observed differences in kinetic slowing under different experimental conditions (Fig. 1), we investigated whether kinetic slowing was associated with voltage-dependent inhibition. Despite the lack of kinetic slowing in perforated-patch recordings (Fig. 1b), somatostatin-mediated inhibition remained strongly voltage-dependent (Fig. 2a). In fact, voltage-dependent inhibition was observed in

whole-cell recordings, perforated-patch recordings, and perforated-patch recordings in the presence of an inhibitor of cGMP-PK (Fig. 2b). These observations dissociate kinetic slowing from voltage-dependent inhibition.

Using the whole-cell recording technique, the time course of somatostatin-mediated Ca^{2+} -current inhibition showed desensitization over time and with repeated application (Fig. 3a). In contrast, inhibition by somatostatin in perforated-patch recordings showed little or no desensitization following several 3-min applications (Fig. 3b). However, perforated-patch recordings made with the inhibitor of cGMP-PK (100 μM Rp-8-CPT-cGMPS) revealed an increase in desensitization. The somatostatin-induced Ca^{2+} -current inhibition disappeared within 2–3 min and was not observed after reapplication of the peptide (Fig. 3c). The broad-range protein kinase inhibitor H-7 (at 100 μM ; $n=4$) had the same effect as Rp-8-pCPT-cGMPS (data not shown). Therefore, somatostatin inhibition of Ca^{2+} current appears to act through an endogenous cGMP-PK that is significantly inhibited or lost when cytoplasmic constituents of the cell are removed during whole-cell recordings. Desensitization following kinase blockade is more rapid than in whole-cell recordings. This may be due to the retention of some cytoplasmic components during whole cell dialysis and to the effects of pipette solution additives (ATP, leupeptin and creatine phosphokinase). In either recording configuration, the inhibitory effect of somatostatin was completely abolished by a 5-hour pretreatment with pertussis toxin (Fig. 3d), which suggests that these pathways act through a G protein.

To test further the hypothesis that a soluble pathway utilizing a cGMP-PK can modulate Ca^{2+} current, both $N^2, 2'-O$ -dibutyl guanosine 3', 5'-cyclic monophosphate (dibutyl cGMP) and a specific activator of cGMP-PK, Sp-cyclic-8-bromoguanosine-3', 5'-monophosphorothioate (Sp-8-Br-cGMPS; ref. 10, and E. Butt and U. Walter, manuscript in preparation), were tested.

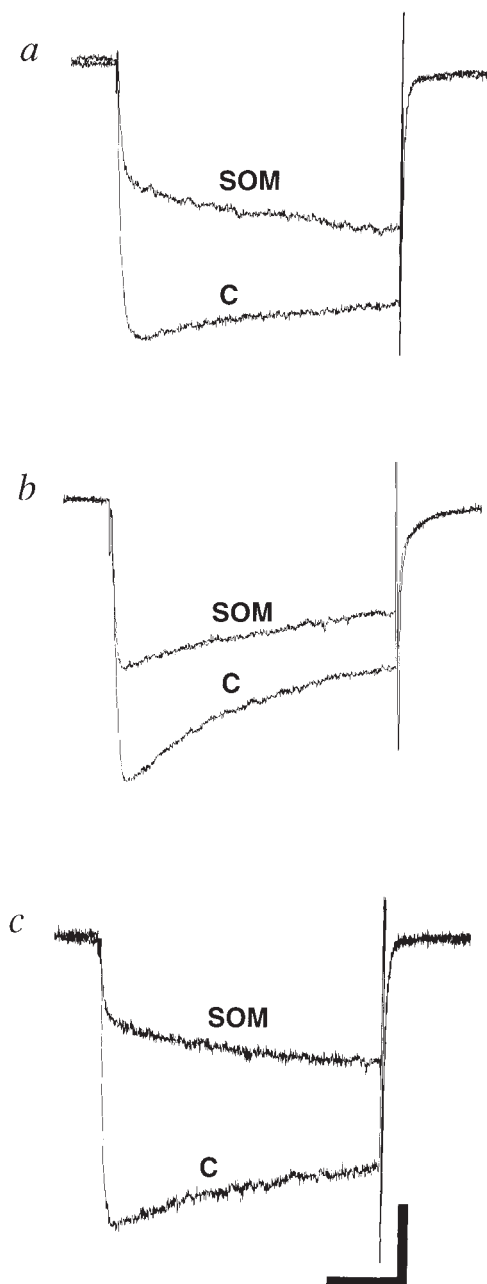
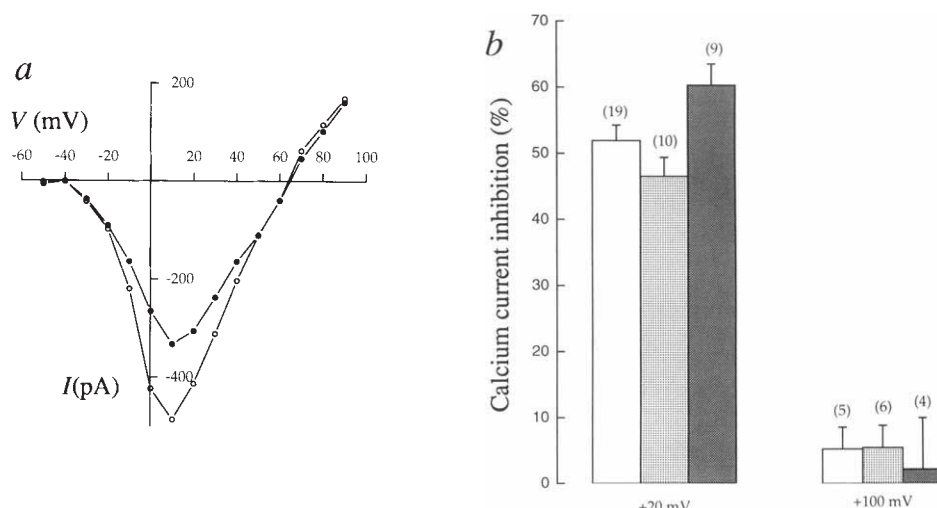


FIG. 1 Ca^{2+} current activated by 100-ms voltage steps from -60 mV to $+20$ mV every 15 s measured before (C) and 15 s after 100 nM somatostatin application (SOM). In whole-cell recordings (a) there is significant kinetic slowing following somatostatin application (SOM) that is not observed in perforated-patch recordings (b). In c, following a 15-min exposure to 100 μM Rp-8-pCPT-cGMPS (a specific inhibitor of cGMP-PK), a perforated-patch recording of somatostatin-mediated inhibition is now coupled with kinetic slowing. Calibration: 200 pA (a); 200 pA (b); 100 pA (c); 30 ms.

METHODS. Stage-40 ciliary ganglion neurons were prepared for culture as described²⁴. Experiments were done after preincubating for 2–6 h in DMEM with 10% chick embryo extract. Cells were bathed in 140 mM NaCl, 20 mM TEA-Cl, 10 mM HEPES, 5 mM glucose, 5 mM KCl, 5 mM CaCl_2 , 2 mM MgCl_2 , 2 μM tetrodotoxin and 5 mM 4-aminopyridine, pH 7.3. Perforated-patch recordings were obtained using pipettes (1.5–3 M Ω) filled with 75 mM Cs_2SO_4 , 55 mM CsCl, 8 mM MgCl_2 , 10 mM HEPES, pH 7.3, plus 400 $\mu\text{g ml}^{-1}$ amphotericin-B²⁵. Within 15 min of seal formation, amphotericin-B induced a low-resistance pathway with a series resistance of 18.6 ± 0.7 M Ω ($n=74$). Recordings of Ca^{2+} current were made for up to 2 h. Currents were activated and acquired using the pClamp (Axon Instruments) software package and an EPC-7 patch clamp amplifier. Currents were 4-pole Bessel-filtered at 3 kHz and digitized at 10 kHz. For whole-cell recordings, pipettes were filled with 120 mM CsCl, 10 mM HEPES, 11 mM EGTA, 5 mM TEA-Cl, 1 mM CaCl_2 , 4 mM MgCl_2 , 14 mM creatine phosphate, 4 mM ATP-Na, 0.3 mM GTP-Na, 0.1 mM leupeptin and 50 units ml^{-1} creatine phosphokinase. In whole-cell recordings, access resistance averaged 6.6 ± 0.5 M Ω , $n=29$. All drugs were applied locally to the cell by a perfusion pipette and washed off with bath solution by a second pipette. The membrane-permeant specific activator and inhibitor of cGMP-PK 1- α (Sp-8-Br-cGMPS and Rp-8-pCPT-cGMPS) were obtained from R. Langhorst and H. Genieser. Other drugs were obtained from Sigma. All were dissolved in aqueous solution except for Sp-8-Br-cGMPS, Rp-8-pCPT-cGMPS, dibutyl cGMP and 8-Br-cAMP, which were dissolved in dimethyl sulfoxide (0.1%). All experiments were at room temperature.

FIG. 2 Voltage-dependence of somatostatin-mediated inhibition of Ca^{2+} current. *a*, Current-voltage relationship for somatostatin-mediated inhibition recorded using perforated-patch recordings. *b*, Summary of the magnitude of somatostatin-mediated Ca^{2+} current inhibition (expressed as per cent inhibition $\pm$ s.e.m.) at +20 mV and +100 mV for whole-cell recordings (open bars), perforated-patch recordings (light shaded bars) and perforated-patch recordings in the presence of Rp-8-pCPT-cGMPS (dark shaded bars). As somatostatin-mediated inhibition of calcium current showed significant desensitization when measured with the whole-cell technique, or in the presence of the specific cGMP-PK inhibitor, the magnitude of inhibition was measured at +20 mV and +100 mV by a voltage pulse protocol that tested only to these two potentials, with a 1-s interval every 15 s. Control Ca^{2+} currents tested to +20 mV twice, with the same 1-s interval, were not significantly different from one another (not shown). Data are expressed as

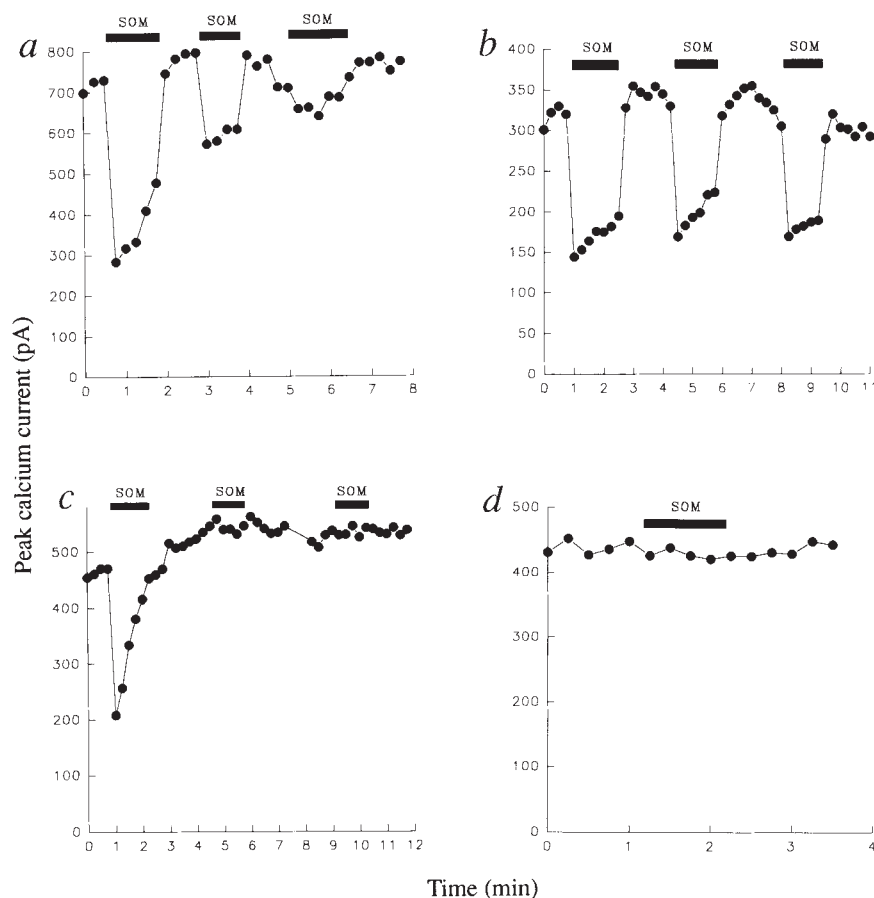


the per cent inhibition of control Ca^{2+} current. Data obtained with single test pulses to +20 mV are also included. Numbers in parentheses indicate the number of cells tested.

In perforated-patch recordings, 100 μM dibutyl cGMP alone mimicked the effects of somatostatin in 7 of 7 cells tested. 100 μM Sp-8-Br-cGMPS also mimicked somatostatin inhibition in a manner indistinguishable from that produced by dibutyl cGMP, but only in 3 of 7 cells tested. Calcium current inhibition mediated by either dibutyl cGMP or the activator Sp-8-Br-

cGMPS was persistent over the time course of the application (Fig. 4a) and did not display kinetic slowing ($45 \pm 2.4\%$ inhibition at the peak; $37 \pm 3.2\%$ at the end of the pulse, $n=11$; Fig. 4b). Moreover, dibutyl cGMP and somatostatin did not have additive effects when given in either application order ($n=10$). Results obtained using perforated-patch recordings were in con-

FIG. 3 Time course of somatostatin-mediated inhibition of Ca^{2+} current. *a*, Using whole-cell patch recordings, repeated 2–3-min applications of somatostatin (SOM) resulted in an inhibition of Ca^{2+} current that showed some desensitization over time. *b*, Somatostatin-mediated inhibition recorded with perforated-patch recordings resulted in persistent and reproducible inhibition of Ca^{2+} current with little or no desensitization. *c*, Following a 15-min exposure to an inhibitor of cGMP-PK (100 μM Rp-8-pCPT-cGMPS), perforated-patch recording of somatostatin-mediated inhibition was transient and could not be observed after repeated applications. *d*, A 5-h pretreatment with 200 units ml^{-1} pertussis toxin completely blocked the somatostatin effect. In *a–d*, the duration of exogenous somatostatin application is indicated by bars. For all experiments, the effects of somatostatin were tested every 15 s. Using this stimulus paradigm, we did not detect differences in the onset of the inhibition when measured using whole-cell or perforated-patch techniques. The Rp-8-pCPT-cGMPS inhibitor did not have any effect ($n=5$) on control Ca^{2+} currents. In perforated-patch recordings, peak Ca^{2+} current averaged 686 ± 93 pA ($n=11$) in cGMP-PK inhibitor-treated cells. This was not significantly different from current magnitudes measured in untreated cells recorded with perforated-patch (617 ± 50 pA, $n=25$) or whole-cell techniques (762 ± 56 , $n=22$). Although there was a significant difference in the inactivation of control Ca^{2+} current in whole-cell ($13.8 \pm 1.9\%$) versus perforated-patch ($39 \pm 3.1\%$) recordings, and apparent changes in activation kinetics (kinetic slowing) could be partially obscured by differences in inactivation, this complication was not present when kinetic slowing was compared in perforated-patch recordings made in the presence and absence of the inhibitor of cGMP-PK.



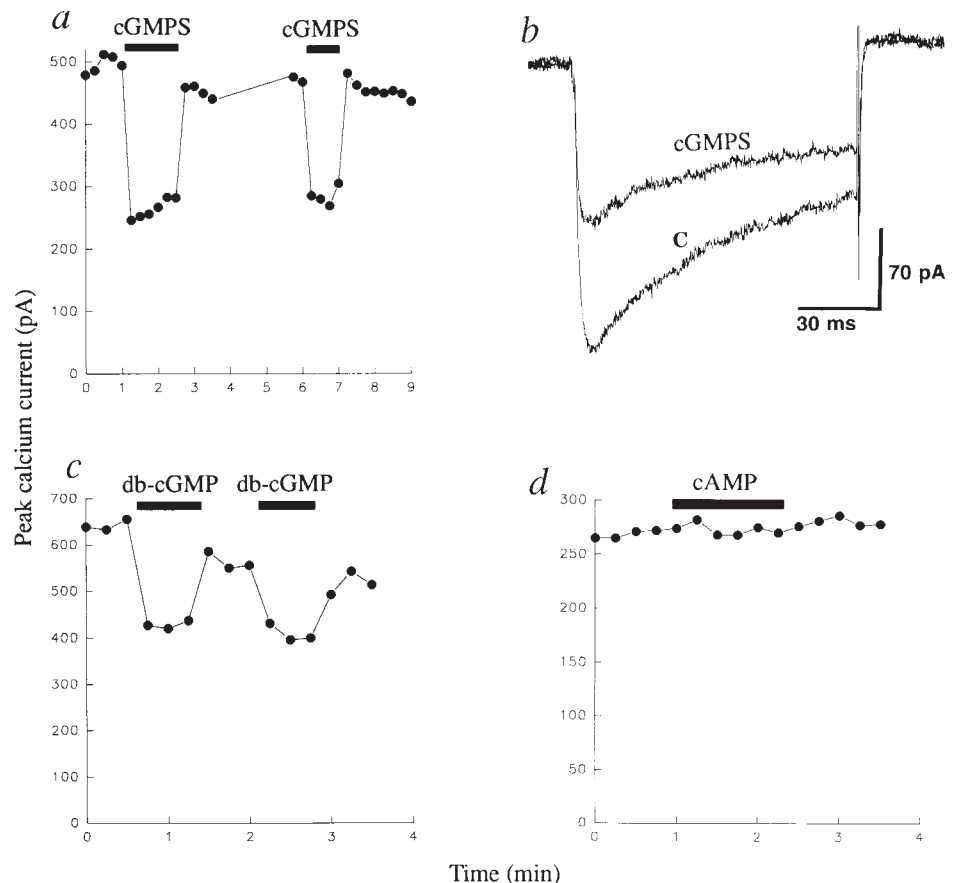


FIG. 4 Effects of an activator of cGMP-PK (Sp-8-Br-cGMPS), dibutyl cGMP, and 8-Br-cAMP on Ca^{2+} current. *a*, In a perforated-patch recording, the magnitude and time course of 100 μM Sp-8-Br-cGMPS-mediated inhibition (cGMPS) mimicked the somatostatin effect on Ca^{2+} current (compare Fig. 3*b*). *b*, Comparison of a control current (C) and the effect of inhibition by Sp-8-Br-cGMPS (cGMPS) demonstrated that the Sp-8-Br-cGMPS-mediated inhibition of Ca^{2+} current occurred without any changes in Ca^{2+} -current activation kinetics. Using whole-cell recordings, 100 μM dibutyl-cGMPS (db-cGMP) had a significantly smaller effect (*c*) than in perforated-patch recordings (see text), and the activator of cGMP-PK (Sp-8-Br-cGMPS) had no effect (not shown). *d*, In a perforated-patch recording, 100 μM 8-Br-cAMP (cAMP) was without effect on Ca^{2+} current.

trast to results obtained using whole-cell recordings, where Sp-8-Br-cGMPS was without effect ($n=7$) and dibutyl cGMP had a significantly ($P<0.005$) smaller effect ($33.3\pm 2.9\%$, $n=8$; Fig. 4*c*). In either recording configuration, 100 μM 8-bromoadenosine 3', 5'-cyclic monophosphate (8-Br-cAMP) was without effect ($n=4$; Fig. 4*d*). Therefore, an endogenous cGMP-PK is available for activation in neurons studied with perforated-patch recordings, but less so following dialysis by whole-cell recording methods. In addition, when this kinase was activated by exogenous cGMP analogues in perforated-patch recordings, Ca^{2+} channels were affected to the same degree as when somatostatin was applied (compare Figs 3*b* and 4*a*).

It is possible that the somatostatin receptor initiates two cascades, both acting through a G protein, that target the Ca^{2+} channel: a membrane-delimited pathway that initiates the Ca^{2+} -current inhibition directly, and a cGMP-dependent protein kinase pathway that maintains the inhibition. This could occur through two types of G proteins¹¹, or through a single G protein that influences two different effectors, as has been demonstrated

for G_s in cardiac cells¹². In either case, the kinase supports and maintains the direct membrane-delimited G-protein action triggered by the somatostatin receptor. As cGMP analogues can mimic somatostatin-mediated inhibition, but are not additive, perhaps the membrane-delimited and soluble pathways are redundant, and act on the same effector. This kinase-mediated maintenance of the Ca^{2+} -current inhibition overrides a type of desensitization that occurs in the absence of cGMP-PK activity. Peptide-mediated inhibition that proceeds through a membrane-delimited pathway in the absence of a kinase often results in kinetic slowing^{1,2,6,7,13-19}. In chick ciliary ganglion neurons, kinetic slowing appears coupled to somatostatin-mediated inhibition that desensitizes. This work alters a long-standing hypothesis that neuropeptide-mediated inhibition of Ca^{2+} -current occurs in the absence of any soluble cytoplasmic component^{3,18-22}. Sorting out the details of these pathways that link neurotransmitter and peptide receptors to ion channels is critical to our understanding of the regulation of transmitter release²³. □

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A 13-amino-acid motif in the cytoplasmic domain of FcγRIIB modulates B-cell receptor signalling

Tatsushi Muta, Tomohiro Kurosaki,
Ziva Misulovin, Mercedes Sanchez,
Michel C. Nussenzweig & Jeffrey V. Ravetch

Nature 368, 70–73 (1994)

In this letter we neglected to mention in our citation of ref. 13 that Amigorena *et al.*¹ had previously expressed in IIA1.6 cells deletion mutants of mFcγRII-B1 and B2 that did or did not include the 13-amino-acid cytoplasmic domain motif analysed in our report. The ability of these mutants to attenuate mIg-induced B-cell activation was correlated with the presence of this region, suggesting its functional involvement in regulating the activation process. It has also been brought to our attention that Choquet *et al.*² used this approach to suggest that the regulatory activity could be localized to the YSLL motif contained within the 13-amino-acid region and that the inhibition of mIg-induced activation by mFcγRII affects the influx of extracellular Ca²⁺. We apologize for these oversights. □

1. Amigorena, S. *et al.* *Science* **256**, 1808–1812 (1992).
2. Choquet, D. *et al.* *J. Cell Biol.* **121**, 355–363 (1993).

CORRECTION

Laser action in strongly scattering media

N. M. Lawandy, R. M. Balachandran,
A. S. L. Gomes & E. Sauvain

Nature 368, 436–438 (1994)

WE omitted to acknowledge that A.S.L.G. was partially funded by the CNPq Brazilian Agency during this work, undertaken while on sabbatical leave from the Departamento de Física, Universidade Federal de Pernambuco, Recife PE 50730, Brazil. □

ERRATUM

Soil acidification and nitrogen saturation from weathering of ammonium-bearing rock

Randy A. Dahlgren

Nature 368, 838–841 (1994)

THE elemental pool sizes shown on the y-axis of Fig. 2 (right panel) of this letter should have been given in units of kg ha⁻¹, and not 'Mg ha⁻¹' as published. □

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Genetics business booming yet uncertain

Helen Gavaghan

For all their high profile, US biotechnology companies in genetics do not yet seem to offer a stable career, because of uncertainties about corporate performance. Meanwhile, attempts are being made to assess the future shape of genetic counselling services, and to attract non-biologists to genome research.

THE career openings for geneticists and biochemists in research-related areas fall in three categories — industry, clinical and 'academic' research. What are the relative prospects?

Industry

Most of the newly formed companies hoping to develop commercial products from research into the human genome face a tough time raising capital to finance their efforts.

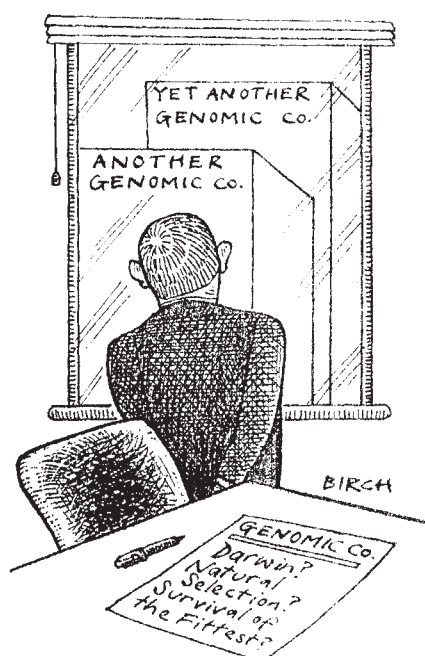
In the past two years, scientists, in alliance with venture capitalists, have branched out from the academic world to form 'genomic' companies. These companies are the latest wave of start-ups in the biotech industry, and they aim to turn basic genome research into diagnostic kits and drug therapies. In general, the genomic companies' products — and therefore profits — lie five to ten years in the future, making them heavily reliant on capital investment to continue their research.

Peter Drake, director of equity research for Vector Securities International, an investment bank specializing in biomedical and health-care companies, says: "To get capital today, companies have to be very creative. It's as ugly out there as I have ever seen it."

Drake believes that the formation of new genomic companies has peaked and that there will be a wave of mergers, acquisitions and closures. Doug Rogers, a general partner in the venture capital company Helix Venture says: "There have been too many genomic companies formed in the past two or three years; we are going to see a huge shake-out."

For scientists contemplating a move to industry, such a shake-out carries real risks. As Rogers points out: "In the antediluvian days of the early 80s, some biotech start-ups had difficulties; Amgen more than once could not meet its payroll." Nevertheless, the financial rewards for the brave are lucrative compared with those in academic life. John Hawkins, managing director of the executive recruitment agency Russell Reynolds Associates fills top jobs in the industry, carrying salaries of between \$150,000 and \$800,000.

For Rogers, the future of genomic companies hinges on whether or not they will attract the best creative scientists. "My



fear is that the truly brilliant guys will stay in basic science." To make breakthroughs, Rogers argues, leading scientists need to be involved on a day-to-day basis, not merely as advisers or on the board of several companies. "The question is — will the capital market run out before these companies convince the corporate world of the value of what they are doing?"

The uncertain outlook for new genomic companies specifically (and the biotech industry in general) stems from the capital investors' general gloom about the health-care industry. There is a continuing reorganization in the way pharmaceutical companies sell drugs, coupled with uncertainty about the impact of health-care reform. Added to this, says Drake, investors always see the biotech industry as risky, and any investment will be in the established biotech companies with products, such as Genentech, Biogen, Amgen and Genzyme.

Another difficulty affecting the new genomic companies' prospects for capital is the disappointment of biotech industry watchers. At the beginning of the year, these market gurus anticipated that a spectrum of products at the clinical trial stage would soon be ready to market. That

optimism has evaporated as clinical trials have failed to prove the efficacy of a variety of products.

"In the past two years," says Rogers, "there have been miscalculations stemming from a failure to understand the nature of clinical trials and a belief that if something was in a phase II trial, it would proceed to phase III [the final stage before approval by the Food and Drug Administration of a new drug]."

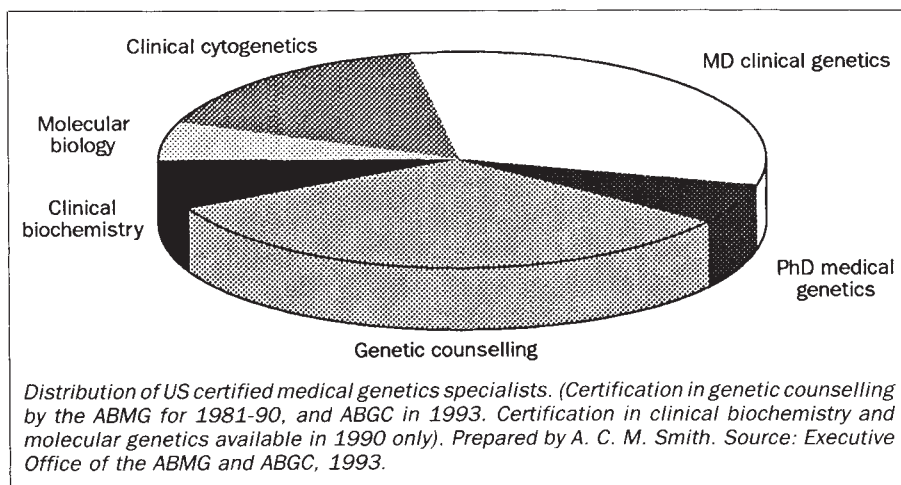
To avoid such miscalculations, Wall Street needs more analysts with an understanding of the science, the regulatory process and business. Says Rogers: "there are too few people on Wall Street interpreting the science for the lay people with the money, and people are trading on how they feel about the economy, not on an understanding of a company."

One genomics company, notable in that it is well capitalized, is Human Genome Services of Bethesda, Maryland. "For a biotech company to succeed," says Drake, "it must have people, capital and technology; HGS has all three."

The company was established two years ago with \$10 million from the HealthCare Investment Corporation. It realized a further \$31 million by selling 20 per cent of its stock and has struck a deal with Smith-Kline Beecham for \$125 million over three years in return for the genetic information it is collecting. The company's strong scientific base comes from a close association with the non-profit Institute for Genomic Research, founded by Craig Venter. (Venter developed important sequencing technology while at the National Institutes of Health.)

Venter's institute employs about 80 people, most of whom are scientists and laboratory technicians. Venter says that the institute should be able to pay their salaries for the next decade. (Their research is paid for by a mixture of private and federal money.) About a third of the technical and scientific staff are specialists in computing or informatics. For the field of genomics to be successful, Venter is convinced of the importance of these specialties. "There is not currently a sufficiently large pool to draw from," he says, "yet computing will be key to molecular biology, computing is how we are going to develop new biological hypotheses."

Whatever the expertise of the scientist,



however, a move into industry no longer means that one cannot return to the less harried halls of academic institutions. Says Venter: "When I left graduate school in 1975, you made a lifetime choice — industry or academia — though there were exceptions. Genentech changed all that. All of a sudden, there was this quality research coming out of a biotech company".

Clinical genetics

Courses in medical genetics are finding it hard to attract newly qualified MDs, according to Victor McKusick, a professor of medical genetics at the Johns Hopkins University in Baltimore, Maryland, and a pioneer of the field of genetic medicine.

In the coming decade, that situation will change dramatically as the Human Genome Project identifies new disease genes and more tests become available to predict whether a patient is likely to develop a particular disease later in life.

McKusick attributes the shortfall in MDs attracted to medical genetics to its being a new field and to lower salaries than for, say, surgeons. Maimon Cohen, president of the American Society of Human Genetics (ASHG), says: "It is true, we are not filling the courses, but I think it is principally because it is only for the past two years that medical genetics has been accredited as a specialty."

In the past, specialists in medical genetics have belonged to groups concerned with other specialties. "We have had no real support slots," says Cohen. The medical schools are aware of this, and are now beginning to change. "But this is an inauspicious time for changes," he says, "with clinical funds shrinking."

MDs represent only one part of the overall change in medical genetics. The whole field is in flux, with a massive future demand anticipated for all types of health-care professionals trained in genetics. Yet nobody has assessed the future needs in the field. Says Cohen: "We have no idea about what's going on, where we are, where we will be, the need for training and

placements at all levels, and the impact on the field of commercial ventures."

For this reason, the ASHG, together with the Council of Regional Networks in Genetics, is conducting a major survey of people in existing services to decide what recommendations should be made for the future. The survey, paid for by the Genetic Diseases Branch of the Institute of Maternal and Child Health, is now being analysed. Even when the analysis is complete, it will be difficult to make recommendations, given the proposed health-care reforms. The most basic question is whether genetic services will be part of the basic health-care package.

Even without the results of the survey, one need is clear, according to Ann Smith, one of the researchers involved in the design and analysis of the survey, and that is the need for genetic counsellors. The counsellors' role is to help patients to understand and to come to terms with diagnoses of genetic disease or information about the likelihood of giving birth to a child carrying a gene that will cause disease. In its report *Assessing Genetic Risk*, the Institute of Medicine said: "Anyone who is offering genetic testing must provide appropriate genetic counselling and education prior to testing." The first course for genetic counselling was founded in 1969, and each year about a hundred people graduate with a master's degree in counselling. Most courses have only four or five places and about a hundred applicants, according to Barbara Biesecker, genetics counsellor at the National Center for Human Genome Research. "Ideally," says Biesecker, "applicants will have a double major in genetics and psychology."

In the United States today, there are about a thousand genetics counsellors, not enough to cope with the predicted increase in demand for their services.

Academic science and training

A draft proposal from the National Center for Human Genome Research (NCHGR) for training between 1995 and 2000 notes

that scientists have not taken advantage of training funds that the centre has already made available.

The NCHGR's training programme, established in 1990, was intended to train scientists in the interdisciplinary skills of molecular biology, engineering, mathematics, physics and chemistry. Such polymaths were needed, according to the organisers of the Human Genome Project, for the success of genomic research.

The centre had money available to support 600 pre- and postdoctoral students and senior fellows between 1990 and 1995. Given the shortfall in applicants, the centre is now co-funding courses with the National Institute for General Medicine to train students in fields connected with genomic research.

This arrangement will be phased out from September 1995, and in the rest of the decade the centre will continue to concentrate on interdisciplinary training. Nevertheless, there are difficulties. "Getting faculty from non-biology departments to work with faculty from molecular biology departments is very difficult," says the report. It also points out that the NCHGR will have to be very aggressive in persuading institutions strong in both biotechnology and genomics to develop training programmes in genomic science.

In a section dealing with the problems of attracting people to genomic research, the report points out that many molecular biology students perceive genomic research to be boring and repetitive, an attitude that is slowly changing.

In 1992, the centre also established awards to attract non-biologists into genomics in response to the widely expressed opinion of genomic researchers that biologically literate engineers, computer scientists, mathematicians, physicists and chemists are essential for the success of the Human Genome Project.

The awards last for between three and five years, and pay salaries of up to \$50,000, provided that they are in line with the academic institution's pay scale. In addition, recipients can request up to \$20,000 a year towards research expenses. The idea is that at the end of the award, recipients would go on to independent genomic research.

But the report identifies problems in attracting such people to genomics. It points out that there is no clear career path in universities for engineers, physicists and the like who enter genome research. Also, "applications from non-biologists are rarely reviewed fairly by NIH study sections because areas like engineering are not considered science". And even if reviews were always fair, non-biologists have little knowledge of genetics, so are not likely to choose a career in genomics.

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